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(54) Title: BIFUNCTIONAL ANTIBIOTICS

(57) Abstract: Bifunctional antibiotics that target both bacterial RNA and resistance-causing enzymes are disclosed. The A-site of bacterial 16S rRNA serves as the target site for most aminoglycoside antibiotics. Resistance to this class of antibiotics is frequently developed by microbial enzymatic acetylation, phosphorylation or ribosylation of aminoglycosides, modifications that weaken their interactions with the target RNA. Using surface plasmon resonance (SPR), the binding affinity and stoichiometry of various aminoglycosides have been investigated and it was found that neamine, the key pharmacophore of the deoxystreptamine class of aminoglycosides, binds to the A-site in a two to one stoichiometry with a K_d of 10 μM for each binding site. A library of neamine dimers was prepared and their affinities to 16S rRNA A-site were determined by SPR, with K_d= 40 nM for the best dimer (an ~ 10^3-fold increase in affinity). Antibiotic activities of the dimers were determined for several bacterial strains by the Kirby-Bauer method. The most active dimer, based on antibiotic activity, also showed the highest inhibition of *in vitro* translation (IC_{50} = 0.055 μM). The latter assay was developed in order to correlate the relationship between SPR-based affinity and translation inhibition. By these combined methods, transport limitations for the semisynthetic aminoglycosides as well as non-ribosomally based antibiotic activity could be determined. Further analysis of these dimers as substrates for aminoglycoside modifying-enzymes identified a neamine dimer that was a potent inhibitor (K_{is} = 0.1 μM) of the APH(2'') activity of the bifunctional enzyme AAC(6'')-APH(2''), the primary enzyme responsible for high level gentamicin C resistance in several bacterial strains.

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BIFUNCTIONAL ANTIBIOTICS

Description

Technical Field:

The invention relates to bifunctional antibiotics. More particularly, the invention related to bifunctional antibiotics that target bacterial rRNA and inhibit resistance-causing enzymes.

Background:

Deoxystreptamine-based aminoglycosides are a clinically important class of antibiotics that are effective against a broad range of microorganisms (Edson, R. S.; Terrel, C. L. *Mayo Clin. Proc.* **1991**, *66*, 1158). It is believed that aminoglycosides exert their therapeutic effect by interfering with translational fidelity during protein synthesis via interaction with the A-site rRNA on the 16S domain of the ribosome (Moazed, D.; Noller, H. F. *Nature* **1987**, *327*, 389; Purohit, P.; Stern, S. *Nature* **1994**, *370*, 659; Formy, D.; et al. *Science* **1996**, *274*, 1367). Unfortunately, the high toxicity and rapid emergence of high level aminoglycoside resistance have severely limited the usefulness of this class of antibiotics. Numerous aminoglycoside resistance mechanisms have been identified, and enzymatic acetylation, phosphorylation and ribosylation are the primary causes of high level resistance in most clinical isolates (Wright, G. D.; et al. *Adv. Exp. Med. Biol.* **1998**, *456*, 27; Kondo, S.; Hotta, K. *J. Infect. Chemother.* **1999**, *5*, 1; Mingeot-Leclerco, M.-P.; et al. *Antimicrob. Agents Chemother.* **1999**, *43*, 727). Of the modifying enzymes, the acetyl- and phosphotransferases (AAC and APH) have been extensively studied with respect to their specificity (Wright, G. D.; et al. *Adv. Exp. Med. Biol.* **1998**, *456*, 27; Kondo, S.; Hotta, K. *J. Infect. Chemother.* **1999**, *5*, 1; Mingeot-Leclerco, M.-P.; et al. *Antimicrob. Agents Chemother.* **1999**, *43*, 727; Daigle, D. M.; et al. *Chem. Biol.* **1999**, *6*, 99; Azucena, E.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 2317; Patterson, J.-E.; Zervos, M. *J. Rev. Infect. Dis.* **1990**, *12*, 644).

What was needed was a method to tackle the problem of antibiotic resistance. What was needed was bifunctional aminoglycosides that can resist

or inhibit aminoglycoside-modifying enzymes while simultaneously targeting ribosomal RNA.

Summary:

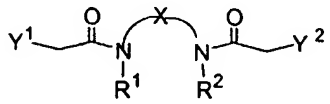
Bifunctional antibiotics are disclosed herein that target both bacterial RNA and resistance causing enzymes. Preferred bifunctional antibiotics are disclosed to be neamine dimers. These neamine dimers represent a new class of aminoglycoside antibiotics that are functionally simpler than previously known aminoglycosides. In addition targeting bacterial RNA, they are also potent inhibitors of the APH(2") activity of the bifunctional AAC(6')-APH(2") enzyme, one of the most clinically significant of the aminoglycoside-modifying enzymes.

Bifunctional antibiotics that target both bacterial RNA and resistance-causing enzymes are disclosed and are demonstrated to provide a method for tackling the problem of antibiotic resistance. The A-site of bacterial 16S rRNA serves as the target site for most aminoglycoside antibiotics. Resistance to this class of antibiotics is frequently developed by microbial enzymatic acetylation, phosphorylation or ribosylation of aminoglycosides, modifications that weaken their interactions with the target RNA. Using surface plasmon resonance (SPR), the binding affinity and stoichiometry of various aminoglycosides have been investigated and it was found that neamine, the key pharmacophore of the deoxystreptamine class of aminoglycosides, binds to the A-site in a two to one stoichiometry with a K_d of 10 μM for each binding site. A library of neamine dimers was prepared and their affinities to 16S rRNA A-site were determined by SPR, with K_d = 40 nM for the best dimer (an ~ 10^3-fold increase in affinity). Antibiotic activities of the dimers were determined for several bacterial strains by the Kirby-Bauer method. The most active dimer, based on antibiotic activity, also showed the highest inhibition of *in vitro* translation (IC_{50} = 0.055 μM). The latter assay was developed in order to correlate the relationship between SPR-based affinity and translation inhibition. By these combined methods, transport limitations for the semisynthetic aminoglycosides as well as non-ribosomally based antibiotic activity could be determined. Further analysis of these dimers as substrates for aminoglycoside modifying-enzymes identified a neamine dimer that

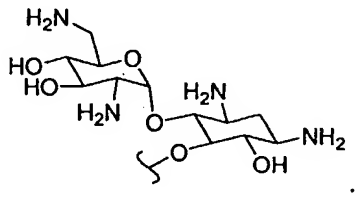
was a potent inhibitor (K_{is} = 0.1 μM) of the APH(2") activity of the bifunctional enzyme AAC(6")-APH(2"), the primary enzyme responsible for high level gentamicin C resistance in several bacterial strains.

One aspect of the invention is directed to a bifunctional antibiotic. The bifunctional antibiotic includes a first and a second pharmacophore and a linkage for linking the first and second pharmacophore. The first and second pharmacophore each has a binding affininty for the A-site of bacterial 16S rRNA sufficient to inhibit translation at clinically effective concentrations. The first and second pharmacophores may be either identical to one another or different from one another. The linkage has a length and structure for enabling the first and second pharmacophore to bind simultaneously to a single A-site of bacterial 16S rRNA. In an improved embodiment of the invention, at least one of the first and second pharmacophores is inhibitory of APH(2") activity with respect to bifunctional enzyme AAC(6')-APH(2"). The inhibitory activity is sufficient, at clinically effective concentrations, to diminish deactivation of the bifunctional antibiotic by the bifunctional enzyme AAC(6')-APH(2").

In one embodiment of this aspect of the invention, the bifunctional antibiotic is represented by the following structure:



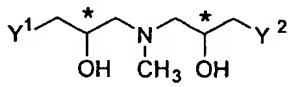
In the above structure, Y^1 and Y^2 are the first and second pharmacophore respectively and are both represented by:



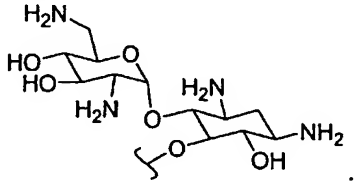
R^1 and R^2 are each independently selected from the group of radicals consisting of -H and $-CH(Ph)CONHCH_2CO_2H$. X is the linkage and is selected from the

group of diradicals consisting of $-(CH_2)_n-$ and $-[(CH_2)_2O(CH_2)_3]_2O$, where $3 \leq n \leq 12$.

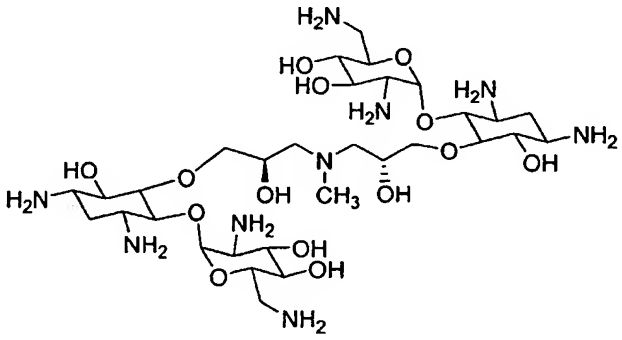
In a second embodiment of this aspect of the invention, the bifunctional antibiotic is represented by the following structure:



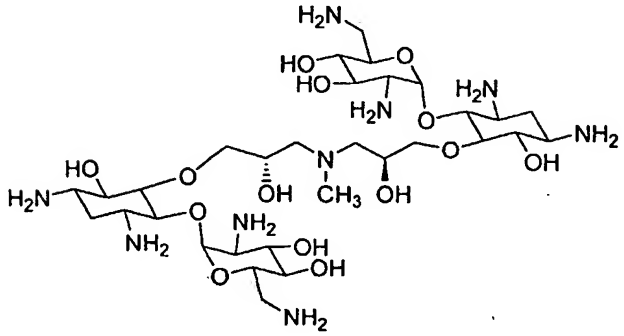
In the above structure, Y^1 and Y^2 are the first and second pharmacophore respectively and are both represented by:



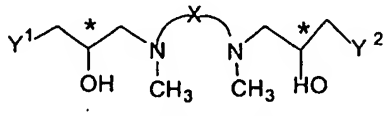
The stereochemistry is either (*S,S*) or (*R,R*). Preferred species of this embodiment include compounds represented by the following structures:



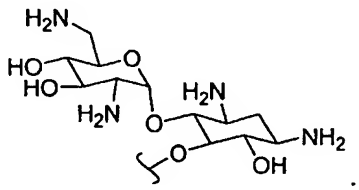
and



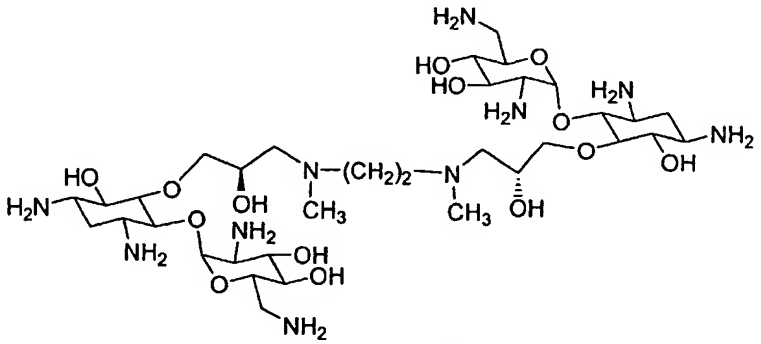
In a third embodiment of this aspect of the invention, the bifunctional antibiotic is represented by the following structure:

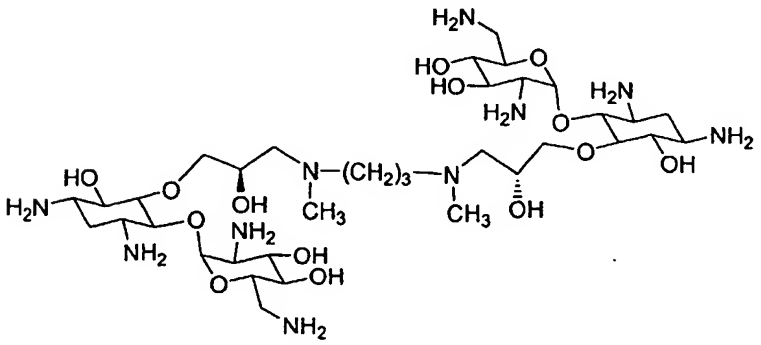


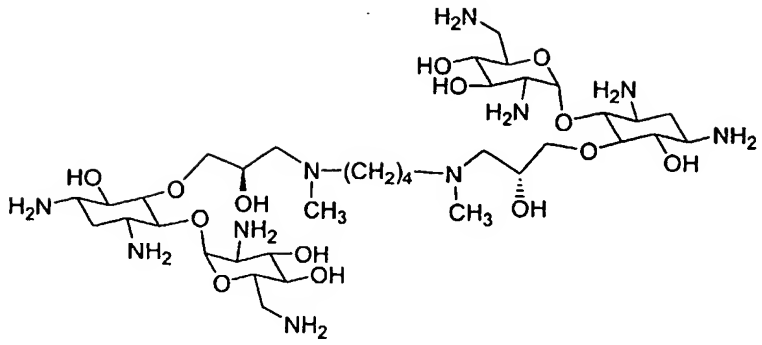
In the above structure, Y^1 and Y^2 are the first and second pharmacophore respectively and are both represented by:

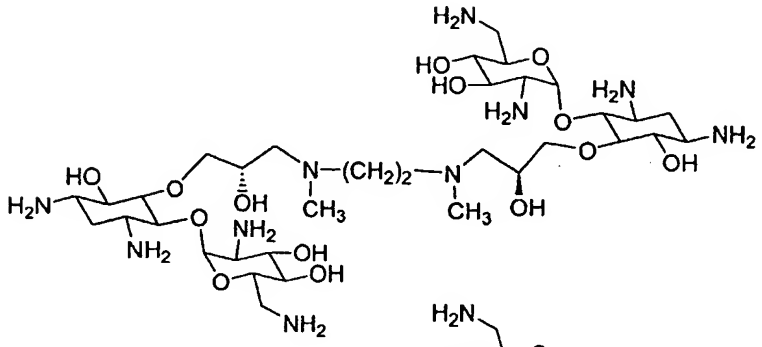


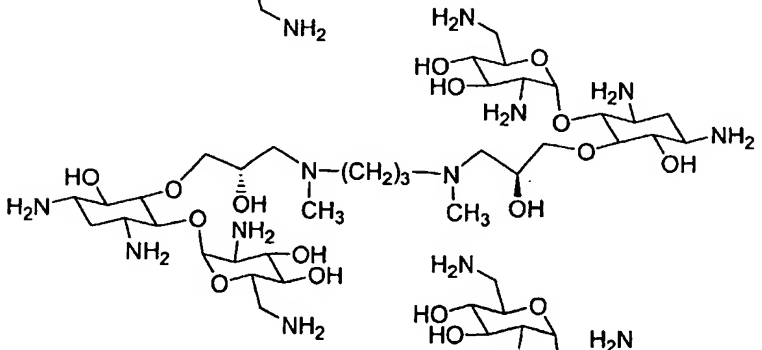
X is the linkage and is selected from the group of diradicals consisting of $-(CH_2)_n-$ and $-[(CH_2)_2]_2O$, where $2 \leq n \leq 4$. The stereochemistry is either (*S,S*) or (*R,R*). Preferred species of this embodiment include compound represented by the following structures:

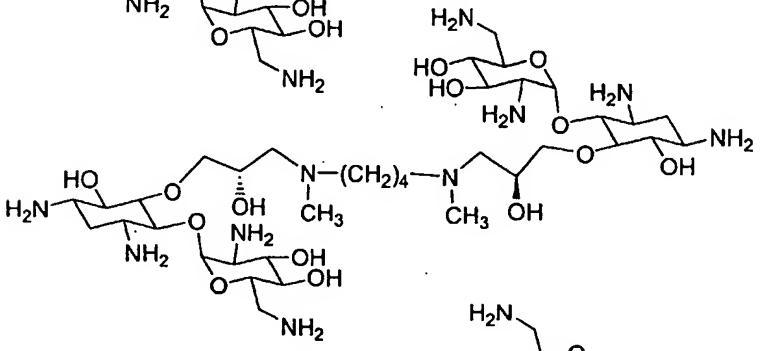


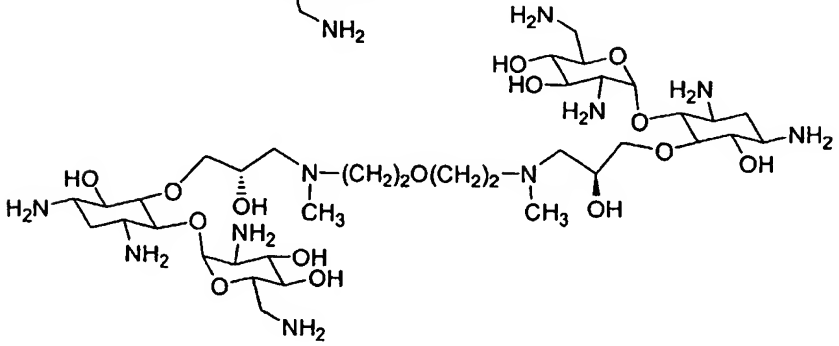




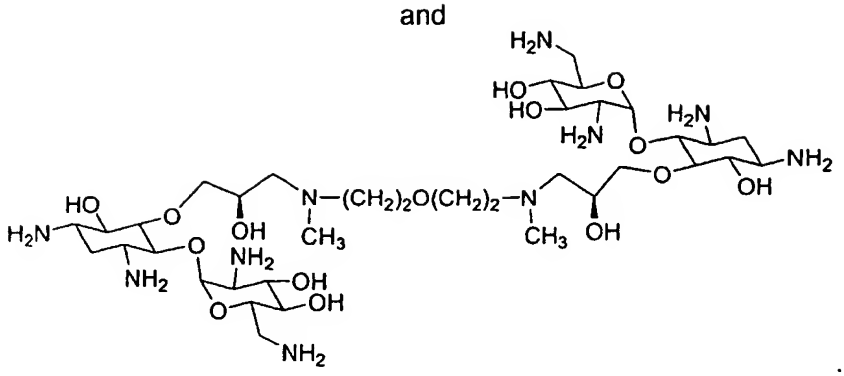








and



Another embodiment of the above invention is directed to a bifunctional antibiotic wherein the first and second pharmacophore are independently selected from the group consisting of neamine, neomycin B, and gentamincin C_1 .

Another aspect of the invention is directed to a process for inhibiting translation within a bacterium having 16S rRNA with an A-site, said process comprising the step of contacting the bacterium with a concentration of any of the bifunctional antibiotics described above sufficient to inhibit translation.

Another aspect of the invention is directed to a process for simultaneously inhibiting translation and APH(2") activity within a bacterium having both 16S rRNA with an A-site and the bifunctional enzyme AAC(6')-APH(2"), said process comprising the step of contacting the bacterium with a concentration of any of the bifunctional antibiotics described above sufficient to inhibit translation and APH(2") activity.

Brief Description of Figures:

Figure 1 illustrates the biotinylated *E. coli* 16S rRNA A-site (AS-wt) rRNA sequence.

Figure 2 illustrates the mode of action of β-hydroxyamine commonly found in aminoglycoside antibiotics.

Figure 3 illustrates is a graph showing a binding isotherm of neamine binding to AS-wt (circles) and control mutants (U1406A, squares; U1485A,

diamonds) for determination of dissociation constants (K_d = inverse slope) and binding stoichiometry (x-intercept).

Figure 4 illustrates an energetic analysis of a bivalent neamine along with a cartoon drawing illustrating how dimers are likely to bind to AS-wt rRNA with high affinity.

Figure 5 illustrates a scheme that shows how the neamine dimers were prepared from a known neamine precursor.

Figure 6 illustrates a graph which demonstrates the relationship between antibiotic activity (MIC, minimum inhibitory concentration) and translation inhibition (IC_{50}).

Figure 7 illustrates the sites of enzymatic modification on neomycin B and gentamicin C_1.

Figure 8 illustsrates a graph showing the results of surface plasmon resonance experiments on neomycin B binding to AS-wt rRNA and mutants.

Figure 9 iillustrates a Scatchard plot for determining dissociation constants (K_d, inverse slope and binding stoichiometry (x-intercept) for the wild type organism.

Figure 10 illutrates an Ugi reaction where four separate components are reacted to produce an amide-linked dimer.

Figure 11 is a table giving the results of the Kirby Bauer test with known compounds and the synthesized dimers.

Figure 12 is a table that shows the minimum inhibitory concentration (MIC, μM) in *E. Coli* ATCC 25922 and *in vitro* translation IC_{50}.

Figure 13 shows tables of the kinetic parameters of neamine and neamine dimers for various aminoglycoside-modifying enzymes.

Detailed Description:

The dissociation constant (K_d) and binding stoichiometry were determined using surface plasmon resonance (SPR) against an immobilized rRNA sequence modeling the A-site of prokaryotic rRNA (Figures 1-4) (Hendrix, M.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 3641; Wong, C.-H.; et al. *Chem. Biol.* **1998**, *5*, 397). The dissociation constants were obtained from equilibrium binding curves through nonlinear curve fitting and were comparable to those obtained using Scatchard analysis. We focused on neamine as it represents the simplest effective aminoglycoside antibiotic and contains the key β-hydroxyamine motif for interaction with the phosphodiester group and the Hoogsteen face of guanine residues in RNA (Figure 2) (Hendrix, M.; et al. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 95). Neamine was found to bind biotinylated AS-wt in a 2:1 complex with a K_d of 10 μM for each binding site (Figure 3). Various dimers of neamine were therefore constructed in order to identify a bivalent aminoglycoside that would bind AS-wt with high affinity (Figure 4), and at the same time resist and and/or inhibit the modifying enzymes due to its unnatural structure (Some aminoglycoside dimers were prepared previously; however, the monomers bind the A-site stoichiometrically: see Michael, K.; et al. *Bioorg. Med. Chem.* **1999**, *7*, 1361; for vancomycin dimers, see Rao, J.; Whitesides, G. H. *J. Am. Chem. Soc.* **1997**, *119*, 10286; Sundram, U. N.; et al. *J. Am. Chem. Soc.* **1996**, *118*, 13107).

Neamine dimers were prepared starting from perbenzyl perazido 5-*O*-carboxyethylneamine (Sucheck, S. J.; et al. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 1080) (see Figure 5), which was prepared from the 5-*O*-allyl precursor (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527). Carboxyethylneamine was distributed into a Quest 210 parallel synthesizer and was activated using a cyclohexylcarbodiimide bound to macroporous polystyrene resin. Two equivalents of resin, one equivalent of acid and 0.4 equivalents of various diamine linkers were utilized to synthesize a library of neamine dimers of variable linker length. The intermediate amides were isolated by filtration and were >95 % pure, as determined by NMR. The resulting dimers were first reduced under Staudinger conditions to convert the azides to amines, which were captured from solution using the resin bound sulfonic acid scavenger MP-TsOH

(Argonaut). The resin was washed and the free amine was released from the resin by elution with 2 M NH_3 in methanol. The resulting amines were debenzylated by hydrogenolysis in the presence of 2 equivalents of acetic acid per amine. The reaction mixture was filtered, concentrated and purified by silica gel chromatography using 8:2:4:5 NH_4OH-$CHCl_3$-*n*-BuOH-EtOH, followed by cation exchange chromatography to give the pure aminoglycosides dimers **4-13**. The amide-linked dimers could also be prepared via Ugi reactions, e.g. dimer **14**, starting from the same perbenzyl perazido 5-O-carboxyethylneamine. This procedure is also directly applicable to parallel synthesis and could be used to increase the molecular diversity of the library.

The dimers with the highest affinity for AS-wt determined by SPR were also the most potent antibiotics, as determined by the antimicrobial assays (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527; Phillips, I; Williams, D. In *Laboratory Methods in Antimicrobial Chemotherapy*; Gerrod, L., Ed.; Churchill Livingstone Press: Edinburg, 1978; pp 3-30) and by IC_{50} of *in vitro* translation (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527). Of this series, the dimers with the highest antibiotic activity, **4** and **6**, showed a K_d of 1.1 μM and 0.8 μM on AS-wt, respectively, ten-fold greater than neamine. Dimers with longer linker lengths had weaker affinities for AS-wt, a trend that correlated with antibiotic activity. Interestingly, all of the dimers continued to display a 2:1 binding stoichiometry, indicating that the increase in affinity is most likely due to an additional favorable (not dimeric) yet weak interaction with AS-wt. Antibiotic activities of dimers **4** and **6** were comparable to neamine, MIC = 31 and 125 μM respectively, against the *E. coli* reference strain (See supplement for antibiotic testing data).

The relatively weak antibiotic activity of these dimers led us to design a flexible and hydrophilic linker by opening the 1,2-propyloxiranes with an amine as shown in Figure 5. The triflate of (*S*)-(-)- and (*R*)-(+)- glycidol (Baldwin, J. J.; et al. *J. Med. Chem.* **1982**, *25*, 931; Schlecker, R.; Thieme, P. C. *Tetrahedron* **1988**, *44*, 3289) was used to alkylate perbenzyl perazido neamine to form epoxides **15** and **16**, respectively. Epoxides **15** and **16** were heated for 16 h in a sealed tube

with excess methylamine to form 1,2-hydroxy amines **17** and **18**, respectively. These hydroxy amines could then be used in an addition reaction with another equivalent of epoxide **15** or **16** to form dimers **19** and **20**, respectively, after deprotection. Epoxides **15** and **16** were also opened with 0.5 equivalents of a *N,N'*-methyldiamines to afford protected dimers **21-28**. *N,N'*-methyldiamine that were not commercially available were readily prepared by a one-pot synthesis via imine formation with a primary diamine and benzaldehyde, alkylation of the intermediate imime with dimethyl sulfate followed by hydrolysis of the alkylimine afforded *N,N'*-methyldiamines in high yield (Devinsky, F.; et al. *Synthesis* **1980**, *4*, 303). The resulting dimers were deprotected as previously described to afford dimers **21-28**. These dimers possessed significantly increased antibiotic activity compared to the amide-linked dimers. Antibiotic activity was greatest with the diaminobutane linker in dimer **27**, which showed a MIC = 6.25 μM against E. coli and K_d = 40 nM (AS-wt) with 1 to 1 stoichiometry (Compound **27** is also effective against other strains, including *P. aeruginosa* ATCC 27853, *P. aeruginosa*, PAO-1, *S. aureus* ATCC 29213 and ATCC 33591-MRSA, and *E. faecalis* ATCC 29212 and is 3 times more effective than tobramycin against the tobramycin-resistant strain of *P. aeruginosa* from cystic fibrosis patients.).

To better understand the relationship between RNA binding and antibiotic activity, inhibition of *in vitro* translation of luciferase gene (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527) was measured as a function of MIC, Figure 6. This analysis was used to validate the target and characterize potential transport limitations for the aminoglycosides, and in vitro translation inhibition is expected to be a better indicator of aminoglycoside selectivity for 16S rRNA compared to binding affinity measurements with the A-site sequences (Hendrix, M.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 3641; Wong, C.-H.; et al. *Chem. Biol.* **1998**, *5*, 397; Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527). A nearly linear relationship between the IC_{50} of translation inhibition and the MIC was observed. This analysis is useful for analyzing structure activity relationships within a similar series of compounds. Compounds falling below the line in Figure 6 may suffer from transport limitation while compounds above the line may act via a fundamentally different mode of action than compounds at or near the line.

Further study of neamime dimers **4**, **6** and **27** using several aminoglycoside-modifying enzymes revealed that the dimers were poor substrates for AAC(6')-Ii and APH(3')-IIIa, responsible for 6'- and 3'- *N*-acetylation and O-phosphorylation, respectively (Wright, G. D.; et al. *Adv. Exp. Med. Biol.* **1998**, *456*, 27; Kondo, S.; Hotta, K. *J. Infect. Chemother.* **1999**, *5*, 1; Mingeot-Leclerco, M.-P.; et al. *Antimicrob. Agents Chemother.* **1999**, *43*, 727). In addition, dimers **4**, **6** and **27** were poor substrates for the AAC(6') activity of the bifunctional aminoglycoside modifying-enzyme AAC(6')-APH(2") (Wright, G. D.; et al. *Adv. Exp. Med. Biol.* **1998**, *456*, 27; Kondo, S.; Hotta, K. *J. Infect. Chemother.* **1999**, *5*, 1; Mingeot-Leclerco, M.-P.; et al. *Antimicrob. Agents Chemother.* **1999**, *43*, 727; Daigle, D. M.; et al. *Chem. Biol.* **1999**, *6*, 99; Azucena, E.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 2317; Patterson, J.-E.; Zervos, M. *J. Rev. Infect. Dis.* **1990**, *12*, 644), and not substrates for the APH(2") activity of AAC(6')-APH(2"). They were in fact potent competitive inhibitors of the APH(2") activity, K_{is} = 0.8 μM for dimer **4**, 0.1 μM for **6** and 0.7 μM for **27**.

Detailed Description of Figures:

Figure 1 shows the biotinylated *E. coli* 16S rRNA A-site (AS-wt) rRNA sequence.
It is this portion of the bacterial RNA on the 16S domain of the ribosome which is bound by the aminoglycosides. This interferes with translational fidelity during protein synthesis.

Figure 2 shows the mode of action of β-hydroxyamine commonly found in aminoglycoside antibiotics. The β-hydroxyamine motif interacts not only with the phosphodiester group but also the Hoogsteen face of guanine residues in RNA.

Figure 3 is a graph showing a binding isotherm of neamine binding to AS-wt (circles) and control mutants (U1406A, squares; U1485A, diamonds) for determination of dissociation constants (K_d = inverse slope) and binding stoichiometry (x-intercept). The binding is sequence selective. The inset in the figure is a Scatchard plot which shows the binding stoichiometry.

Figure 4 is an energetic analysis of a bivalent neamine along with a cartoon drawing illustrating how dimers are likely to bind to AS-wt rRNA with high affinity. Neamine units bind to AS-wt with a K_d of 10 μM per binding site. Addition of the proper linker would enable the unnatural dimer to bind with much higher affinity and resist modifying enzymes because of its unnatural structure.

Figure 5 is a scheme that shows how the neamine dimers were prepared from a known neamine precursor. The starting material is perbenzyl perazido 5-*O*-carboxyethylneamine which is prepared from the 5-*O*-allyl precursor. A variety of diamines were chosen to form a diamide linker to the neamine units. Dimer 14 was synthesized using the Ugi reaction. Four separate components are added during this synthetic procedure. Synthetic steps from the neamine expoxides are shown at the bottom of the scheme. Simple nucleophilic opening of the epoxide ring generates the dimers from a primary amine or a primary diamine precursor.

Figure 6 is a graph which demonstrates the relationship between antibiotic activity (MIC, minimum inhibitory concentration) and translation inhibition (IC_{50}). The compounds above the line do not target RNA and have different modes of antibiotic action, while those to the right of the line exhibit transport limitations. What was measured is the inhibition of *in vitro* translation of the luciferase gene measured as a function of MIC. This analysis was used to validate the target and characterize potential transport limitations for the aminoglycosides, and *in vitro* translation inhibition is expected to be a better indicator of aminoglycoside selectivity for 16S rRNA compared to binding affinity measurements with the A-site sequences. A nearly linear relationship between IC_{50} of translation inhibition and the MIC was observed.

Figure 7 shows the sites of enzymatic modification on neomycin B and gentamicin C_1. *N*-acetylation, phosphorylation and *O*-ribosylation are the major modifications catalyzed by resistance causing enzymes.

Figure 8 is a graph showing the results of surface plasmon resonance

experiments on neomycin B binding to AS-wt rRNA and mutants. The circles are for the wild type organism and the squares and diamonds are for the two different mutants. The binding is sequence selective.

Figure 9 is a Scatchard plot for determining dissociation constants (K_d, inverse slope and binding stoichiometry (x-intercept) for the wild type organism. The binding is sequence selective.

Figure 10 shows an Ugi reaction where four separate components are reacted to produce an amide linked dimer. Compound **14** was synthesized in this reaction to give a linked dimer. 5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzyl-neamine (60 mg, 79 μmol), methyl isocyanoacetate (36 μL, 397 μmol), benzaldehyde (8 μL, 79 μmol), and diaminododecane (8 mg, 40 μmol) were dissolved in a mixture of anhydrous CH_2Cl_2/methanol (1:1, 800 μl). After stirring 48 hours at ambient temperature, the reaction was diluted with ethyl acetate (5 ml). It was then washed with 1M HCl (2 x 5 ml), saturated sodium bicarbonate (2 x 5 ml), and brine (1 x 5 ml). The aqueous extracts were re-extracted with ethyl acetate (2 x 5 ml). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. Flash chromatography (silica gel, gradient hexane to 1:1 hexane/ethyl acetate) yielded protected neamine dimer **14** (21.7 mg, 26%).

Figure 11 is a table giving the results of the Kirby Bauer test with known compounds and the synthesized dimers. The numbers under the test strains are for diameters (mm) of zones of inhibition. All compounds except neomycin and gentamicin were spotted at 200 nmoles/disk; neomycin was spotted at 33 nmoles/disk (30 μg) while gentamicin was spotted at 10 nmole/disk (10 μg). Surface plasmon resonance K_d values for dimers **4-13** is also provided.

Figure 12 is a table that shows the minimum inhibitory concentration (MIC, μM) in *E. Coli* ATCC 25922 and *in vitro* translation IC_{50}. The data from this table is graphed in figure 6 and shows the likely mechanism of action for the antibiotics.

Figure 13 shows tables of the kinetic parameters of neamine and neamine dimers for various aminoglycoside-modifying enzymes. BF refers to the bifunctional enzyme AAC(6')-APH(2"), where the particular activity is indicated. The neamine data were obtained from Daigle, D. M.; et al. *Chem. Biol.* **1999**, *6*, 99.

Experimental Section:

Reactions were performed under inert atmosphere unless otherwise stated. THF and CH_2Cl_2 were distilled under Ar with benzophenone ketyl and CaH_2, respectively. NMR spectra were obtained on a Bruker AMX-400. The sites of enzymatic modification of neomycin and gentamicin that cause drug resistance are shown in Figure 1 (Daigle, D. M.; et al. *Chem. Biol.* **1999**, *6*, 99). Synthesis of biotinylated RNAs and surface plasmon resonance experiments were performed as previously described and K_d values were also calculated as previously described (figure 2) (Hendrix, M.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 3641).

Antimicrobial Testing: The Kirby-Bauer Disk assay was performed as previously described (Hendrix, M.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 3641; Phillips, I.; Williams, D. In *Laboratory Methods in Antimicrobial Chemotherapy*; Gerrod, L., Ed.; Churchill Livingstone Press: Edinburg, 1978; pp 3-30). Reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were obtained as lyophilized pellets (Difco). MIC testing was performed as recommended in the NCCLS Publication M7-A4.

***In vitro* translation assays**: A coupled transcription-translation assay was performed as previously described with luciferase DNA to determine the extent of translational inhibition in the presence of the various aminoglycosides/mimetics (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527). The transcription/translation mixture, or S-30 extract, and the reaction buffers were prepared as described previously with slight modifications (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527). The translation assays were performed

by mixing all of the reagents, various amounts of the compounds to be tested, and the DNA template into a small, RNase-free microcentrifuge tube. The final addition was always S-30 extract, and the reaction was maintained at 21°+/- 1°C in a water bath. The reaction was terminated after 30 minutes by diluting the reaction 10-fold with a luciferase dilution buffer containing 1% Triton X-100. Translation yield was determined by mixing 10µL of the diluted reaction mixture with 50µL of luciferase assay reagent (20mM Tricine, pH 7.8; 15mM $MgSO_4$; 0.1mM EDTA; 33.3mM DTT; 270µM coenzyme A; 470µM luciferin; and 530µM ATP) and monitoring the luminescence with a Turner Designs luminometer. For each assay, points were collected in duplicate, and the full assays were performed at least three times.

5-Ethylcarboxyl-1,3,2',6'-tetraazido-6, 3', 4'-tri-*O*- benzylneamine. (Sucheck, S. J.; et al. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 1080) 5-*O*-Allyl -1, 3, 2',6'-tetraazido-6, 3', 4'-tri-*O*- benzylneamine (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527) (264 mg, 0.340 mmol) was dissolved in 14 mL of 1:1 methanol-dichloromethane and was cooled to -78 °C. Ozone was bubbled through the solution until it became light blue in color. The solution was treated with 272 µL of dimethyl sulfide and was allowed to stir one hour while it warmed to room temperature. The solvents were removed under diminished pressure and the crude aldehyde was taken up in 6 mL of 1:1 carbon tetrachloride-acetic acid. The solution was cooled to 0 °C in an ice bath and 305 mg of sodium chlorite (3.39 mmol) was added in portions over 1 h. The solution was poured into an ice cold $Na_2S_2O_5$ solution, acidified to pH 1 with 0.5 N H_2SO_4, extracted with five 50-mL portions ethyl acetate and dried ($MgSO_4$). The solution was concentrated by co-evaporation with toluene under diminished pressure. The product was purified by silica gel flash column chromatography (3 x 15 cm). Elution with 2:1+1 % hexanes-ethyl acetate-acetic acid afforded the carboxylic acid as a colorless foam: yield 205 mg (80 %); silica gel TLC R_f 0.56 (1:1+1 % hexanes-ethyl acetate-acetic acid); mass spectrum (FAB), m/z 887.1961 $(M + Cs)^+$ ($C_{35}H_{38}N_{12}O_8Cs$ requires 887.1990).

General Procedure for the Synthesis of Neamine Dimers. 5-Ethylcarboxyl-1,

3 ,2', 6'-tetraazido-6, 3' ,4'-tri-*O*-benzylneamine (0.0826 mmol/tube) was dissolved in 1.5 mL/tube of dry dichloromethane and was distributed into a Quest 210 parallel synthesizer (Argonaut Technologies; San Carlos, CA). To each tube was added 143 mg of MP-carbodiimide resin (1.15 mmol/g) (Argonaut Technologies; San Carlos, CA) followed by the diamine (0.0413 mmol/tube). The solutions were agitated for 16 hours, filtered and concentrated under diminished pressure to obtain the dimers as colorless foams.

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)-propylamide, Protected Dimer 4.** Yield: 16.9 mg (26 %); silica gel TLC R_f 0.54 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1569.6495 $(M + Na)^+$ ($C_{73}H_{82}N_{26}O_{14}Na$ requires 1569.6401).

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)butyl-amide, Protected Dimer 5.** Yield: 25.9 mg (40 %); silica gel TLC R_f 0.54 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1583.6550 $(M + Na)^+$ ($C_{74}H_{84}N_{26}O_{14}Na$ requires 1583.6558).

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)-pentylamide, Protected Dimer 6.** Yield: 33.1 mg (51 %); silica gel TLC R_f 0.54 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1597.6744 $(M + Na)^+$ ($C_{75}H_{86}N_{26}O_{14}Na$ requires 1597.6714).

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)-hexylamide, Protected Dimer 7.** Yield: 25.8 mg (39 %); silica gel TLC R_f 0.54 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1611.6886 $(M + Na)^+$ ($C_{76}H_{88}N_{26}O_{14}Na$ requires 1611.6871).

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)-heptylamide, Protected Dimer 8.** Yield: 39.6 mg (60 %); silica gel TLC R_f 0.54 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1625.7021 $(M + Na)^+$ ($C_{77}H_{90}N_{26}O_{14}Na$ requires 1625.7027).

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)-octylamide, Protected Dimer 9.**. Yield: 31.7 mg (47 %); silica gel TLC R_f 0.54 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1639.7137 (M + Na)+ ($C_{78}H_{92}N_{26}O_{14}Na$ requires 1639.7184).

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)-nonylamide, Protected Dimer 10.** Yield: 29.4 mg (44 %); silica gel TLC R_f 0.59 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1653.7423 $(M + Na)^+$ ($C_{79}H_{94}N_{26}O_{14}Na$ requires 1653.7340).

***N,N'*-1,3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)decyl-amide, Protected Dimer11.** Yield: 36.7 mg (54 %); silica gel TLC R_f 0.63 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1667.7483 $(M + Na)^+$ ($C_{80}H_{96}N_{26}O_{14}Na$ requires 1667.7497).

***N,N'*-1, 3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3',4'-tri-*O*-benzylneamine)-dodecylamide, Protected Dimer 12.** Yield: 34.2 mg (50 %); silica gel TLC R_f 0.65 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1695.7802 $(M + Na)^+$ ($C_{82}H_{100}N_{26}O_{14}Na$ requires 1695.7810).

***N,N'*-1,3-bis(5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)-4, 7, 10-trioxotetradecylamide, Protected Dimer 13.** Yield: 32.5 mg (47 %); silica gel TLC R_f 0.26 (1:1 hexanes-ethyl acetate); mass spectrum (MALDI-FTMS), m/z 1715.7432 $(M + Na)^+$ ($C_{80}H_{96}N_{26}O_{17}Na$ requires 1715.7344).

General Procedure for the Azide Reduction of Neamine Dimers. The *N,N'*-bis(5-ethyl-carboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-*O*-benzylneamine)alkylamides were dissolved in 1.5 mL/tube of dry THF and were distributed into a Quest 210 parallel synthesizer. To each tube was added 150 µL of water followed by 15 µL of 1 N NaOH solution. To the resulting solutions were added 10 equivalents of 1 M trimethylphosphine in THF for each tube. The solutions were agitated for 16 hours and 100 mg/tube of MP-TsOH

resin (1.32 mmol/g) (Argonaut Technolo-gies; San Carlos, CA) was added. The solutions were allowed to agitate for 2 hours and were washed with three 10-mL portions of methanol. The resin bound amines were released from the resin by washing the resin with two 5-mL portions of 2 N ammonia in methanol. The solutions were concentrated under diminished pressure to obtain the amines as light yellow syrups. The amines were subjected to hydrogenolysis conditions without further characterization.

General Procedure for the Hydrogenolysis of Neamine Dimers. The *N,N'*-bis(5-ethyl-carboxyl-6, 3', 4'-tri-*O*-benzylneamine)alkylamides were dissolved in 1 mL/vial of glacial acetic acid. To each vial was added 50 µg of 20 % $Pd(OH)_2/C$ (Degussa type) and the solutions were placed under 1 atm of H_2. The solutions were stirred for 16 hours and were concentrated under diminished pressure. The deprotected dimers were purified by flash chromatography on silica gel (1 x 15 cm). Elution with 8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol afforded the dimers as a colorless glasses. The dimers were resuspended in water and applied to Dowex 50WX4-50 H^+ and washed with 5 mL of water. The dimers were eluted with 3 % ammonium hydroxide to obtain the dimers as colorless foams after lyophilization.

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)propylamide (4).** Yield: 0.9 mg (10 %); silica gel TLC R_f 0.32 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 821.4355 $(M + Na)^+$ ($C_{31}H_{62}N_{10}O_{14}Na$ requires 821.4345).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)butylamide (5).** Yield: 1.4 mg (10 %); silica gel TLC R_f 0.32 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 835.0000 $(M + Na)^+$ ($C_{32}H_{64}N_{10}O_{14}Na$ requires 835.4501).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)pentylamide (6).** Yield: 0.9 mg (5.2 %); silica gel TLC R_f 0.32 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z

849.4668 $(M + Na)^+$ ($C_{33}H_{66}N_{10}O_{14}Na$ requires 849.4658).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)hexylamide (7).** Yield: 1.4 mg (10 %); silica gel TLC R_f 0.37 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 863.4838 $(M + Na)^+$ ($C_{34}H_{68}N_{10}O_{14}Na$ requires 863.4814).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)heptylamide (8).** Yield: 1.7 mg (8.1 %); silica gel TLC R_f 0.37 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 855.5173 $(M + H)^+$ ($C_{35}H_{71}N_{10}O_{14}$ requires 855.5151).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)octylamide (9).** Yield: 4.4 mg (26 %); silica gel TLC R_f 0.58 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 891.5131 $(M + Na)^+$ ($C_{36}H_{72}N_{10}O_{14}Na$ requires 891.5127).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)nonylamide (10).** Yield: 2.4 mg (15 %); silica gel TLC R_f 0.74 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 883.5472 $(M + H)^+$ ($C_{37}H_{75}N_{10}O_{14}$ requires 883.5464).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)decylamide (11).** Yield: 13 mg (13 %); silica gel TLC R_f 0.74 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 897.5583 $(M + H)^+$ ($C_{38}H_{77}N_{10}O_{14}$ requires 897.5621).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)dodecylamide (12).** Yield: 0.7 mg (3.7 %); silica gel TLC R_f 0.79 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol-butanol); mass spectrum (MALDI-FTMS), m/z 947.5729 $(M + Na)^+$ ($C_{40}H_{80}N_{10}O_{14}Na$ requires 947.5753).

***N,N'*-1,3-bis(5-Ethylcarboxyl-neamine)-4,7,10-trioxotetradecylamide (13).**

Yield: 2.2 mg (12 %); silica gel TLC R_f 0.79 (8:2:5:4 30 % ammonium hydroxide-chloroform-ethanol- butanol); mass spectrum (MALDI-FTMS), m/z 1715.7432 $(M + Na)^+$ ($C_{38}H_{76}N_{10}O_{17}Na$ requires 1715.7344).

Protected Neamine Dimer 14. For a schematic Ugi reaction, see Figure 3. In a representative example, 5-Ethylcarboxyl-1, 3, 2', 6'-tetraazido-6, 3', 4'-tri-O-benzylneamine neamine (60 mg, 79 µmol), methyl isocyanoacetate (36 µL, 397 µmol), benzaldehyde (8 µL, 79 µmol), and diaminododecane (8 mg, 40 µmol) were dissolved in a mixture of anhydrous CH_2Cl_2/methanol (1:1, 800 µl). After stirring 48 hours at ambient temperature, the reaction was diluted with ethyl acetate (5 ml). It was then washed with 1M HCl (2 x 5 ml), saturated sodium bicarbonate (2 x 5 ml), and brine (1 x 5 ml). The aqueous extracts were re-extracted with ethyl acetate (2 x 5 ml). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. Flash chromatography (silica gel, gradient hexane to 1:1 hexane/ethyl acetate) yielded protected neamine dimer **14** (21.7 mg, 26%). HRMS (FAB) calcd for $C_{104}H_{122}N_{28}O_{20}$ $(M + Cs)^+$ 2215.8445, found 2215.8587.

Neamine Dimer 14. Protected neamine dimer **14** (21.4 mg, 10 µmol) was suspended in ethanol (250 µl). Anhydrous hydrazine (3.2 µl, 100 µmol) was added, followed by Raney nickel (~10 mg) that had been washed thoroughly with ethanol. The reaction was stirred overnight at ambient temperature, then filtered through a plug of Celite and concentrated. The resulting residue was dissolved in H_2O/AcOH (1:1, 0.04 M). $Pd(OH)_2$/C (~10 mg, Degussa type) was added and the reaction stirred under a H_2 atmosphere (balloon) overnight. The reaction was filtered through a plug of Celite and lyophilized. Purification was accomplished on CG-50 cation exchange resin, eluting with a gradient of 0 to 40 % NH_3/H_2O, to give neamine dimer **14** (3.4 mg, 26 %). ^1H NMR (500 MHz, D_2O) δ 7.48-7.36 (10H, bs), 5.71 (2H, d, J = 4 Hz), 4.34 (1H, d, J = 16 Hz), 4.15 (1H, d, J = 15 Hz), 3.95-3.32 (32H, m), 2.42-2.35 (2H, m), 1.76 (2H, dd, J = 26, 13 Hz), 1.27-0.92 (20H, m); ES-MS (neg) calcd for $C_{60}H_{98}N_{12}O_{20}$ $(M - H)^-$ 1306, found 1306.

Epoxide 15. To 500 mg of perbenzyl-perazido-neamine (Greenberg, W. A.; et al.

J. Am. Chem. Soc. **1999**, *121*, 6527) (0.720 mmol) dissolved in 5 mL of THF was added 31.7 mg of 60 % sodium hydride in paraffin (0.793 mmol). Freshly prepared (*S*)-glycidol triflate (121 mg, 0.793 mmol) was added and the solution stirred overnight at room temperature. The solution was quench with saturated NH_4Cl and partitioned with three 50 mL-aliquots of ethyl acetate. The solution was dried ($MgSO_4$) and concentrated under diminished pressure. The crude epoxide was purified by flash chromatography on silica gel (30 x 150 mm). The pure product was eluted with 6:1 hexanes-ethyl acetate to afford the epoxide **15** as a colorless foam: yield 405 mg (75 %); TLC R_f 0.32 (6:1 hexanes-ethyl acetate); mass spectrum (MALDIFTMS): m/z 775.3038 [M + Na^+] ($C_{36}H_{40}N_{12}O_7Na$ requires 775.3041).

Epoxide 16. To 280 mg of perbenzyl-perazido-neamine (Greenberg, W. A.; et al. *J. Am. Chem. Soc.* **1999**, *121*, 6527) (0.403 mmol) dissolved in 5 mL of THF was added 17.8 mg of 60 % sodium hydride in paraffin (0.444 mmol). Freshly prepared (*R*)-glycidol triflate (65.3 mg, 0.444 mmol) was added and the solution stirred overnight at room temperature. The solution was quench with saturated NH_4Cl and partitioned with three 50 mL-aliquots of ethyl acetate. The solution was dried ($MgSO_4$) and concentrated under diminished pressure. The crude epoxide was purified by flash chromatography on silica gel (30 x 150 mm). The pure product was eluted with 6:1 hexanes-ethyl acetate to afford the epoxide **16** as a colorless foam: yield: 280 mg (92 %); TLC R_f 0.32 (6:1 hexanes-ethyl acetate); mass spectrum (ESI): m/z 775 [M + Na^+] ($C_{36}H_{40}N_{12}O_7Na$ requires 775).

Protected Monomer 17. yield: 50.6 mg (65 %), TLC R_f 0.31 (2:2:96 triethylamine-methanol-dichloromethane); mass spectrum (MALDIFTMS): m/z 784.3616 [M + H^+] ($C_{37}H_{46}N_{13}O_7$ requires 784.3643).

Protected Monomer 18. yield: 52.0 mg (66.7 %); TLC R_f 0.31 (2:2:96 triethylamine-methanol-dichloromethane); (MALDIFTMS), m/z 784.3632 [M + H^+] ($C_{37}H_{46}N_{13}O_7$ requires 784.3643). **Protected Dimer 19.** yield: 33.2 mg (72 %), TLC R_f 0.38 (2:2:96 triethylamine-methanol-dichloromethane); mass spectrum (MALDIFTMS): m/z 1536.6846 [M + H^+] ($C_{73}H_{86}N_{25}O_{14}$ requires 1536.6786).

Protected Dimer 20. yield: 37.5 mg (73.4 %); TLC R_f 0.38 (2:2:96 triethylamine-methanol-dichloro-methane); (MALDIFTMS), m/z 1536.6711 [M + H^+] ($C_{73}H_{86}N_{25}O_{14}$ requires 1536.6785).

Protected Dimer 21. yield: 39.2 mg (74 %), TLC R_f 0.38 (2:2:96 triethylamine-methanol- dichloromethane); mass spectrum (MALDIFTMS): m/z 1593.7404 [M + H^+] ($C_{76}H_{93}N_{26}O_{14}$ requires 1593.7365).

Protected Dimer 22. yield: 29.3 mg (54.9 %), TLC R_f 0.38 (2:2:96 triethylamine-methanol-dichloromethane); (MALDIFTMS): m/z 1607.7503 [M + H^+] ($C_{77}H_{95}N_{26}O_{14}$ requires 1607.7521).

Protected Dimer 23. yield: 29.2 mg (54 %), TLC R_f 0.38 (2:2:96 triethylamine-methanol-dichloromethane); mass spectrum (MALDIFTMS): m/z 1621.7658 [M + H^+] ($C_{78}H_{76}N_{26}O_{14}$ requires 1621.7678).

Protected Dimer 24. yield: 31.2 mg (58 %), TLC R_f 0.38 (2:2:96 triethylamine-methanol-dichloromethane); (MALDIFTMS): m/z 1637.7633 [M + H^+] ($C_{78}H_{97}N_{26}O_{15}$ requires 1637.7627).

Protected Dimer 25. yield: 42.9 mg (90 %); TLC R_f 0.30 (2:2:96 triethylamine-methanol-dichloromethane); (MALDIFTMS), m/z 1593.7299 [M + H^+] ($C_{76}H_{93}N_{26}O_{14}$ requires 1593.7365).

Protected Dimer 26. yield: 37.5 mg (77.9 %); TLC R_f 0.26 (2:2:96 triethylamine-methanol-dichloromethane); (MALDIFTMS), m/z 1607.7531 [M + H^+] ($C_{77}H_{95}N_{26}O_{14}$ requires 1607.7521).

Protected Dimer 27. yield: 8.2 mg (17.0 %), TLC R_f 0.23 (2:2:96 triethylamine-methanol-dichloromethane); (MALDIFTMS), m/z 1621.7526 [M + H^+] ($C_{78}H_{97}N_{26}O_{14}$ requires 1621.7677).

Protected Dimer 28. yield: 8.2 mg (16.7 %); TLC R_f 0.29 (2:2:96

triethylamine-methanol-dichloromethane); mass spectrum (MALDIFTMS): m/z 1637.7633 [M + H^+] ($C_{78}H_{97}N_{26}O_{15}$ requires 1637.7627).

Monomer 17. yield: 4.5 mg (34 %); TLC R_f 0.29; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (MALDIFTMS), m/z 410.2596 [M + H^+] ($C_{16}H_{36}N_5O_7$ requires 410.2609).

Monomer 18. yield: 14.8 mg (47 %); TLC R_f 0.27; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (MALDIFTMS), m/z 432.2421 [M + Na^+] ($C_{16}H_{35}N_5O_7Na$ requires 432.2429).

Dimer 19. yield: 6.3 mg (35 %); TLC R_f 0.21; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (MALDIFTMS), m/z 788.4761 [M + H^+] ($C_{31}H_{66}N_9O_{14}Na$ requires 788.4729).

Dimer 20. yield: 4.9 mg (26 %); TLC R_f 0.24; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (MALDIFTMS), m/z 810.4514 [M + Na^+] ($C_{31}H_{65}N_9O_{14}Na$ requires 810.4543).

Dimer 21. yield: 5.9 mg (57 %); TLC R_f 0.27; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (ESI), m/z 843 [M - H^-] ($C_{34}H_{71}N_{10}O_{14}$ requires 843).

Dimer 22. yield: 8.1 mg (99 %); TLC R_f 0.29; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (ESI), m/z 859 [M + H^+] ($C_{35}H_{75}N_{10}O_{14}$ requires 859).

Dimer 23. yield: 2.6 mg (16 %); TLC R_f 0.27; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (MALDIFTMS), m/z 895.5439 [M + Na^+] ($C_{36}H_{76}N_{10}O_{14}$ requires 895.5435).

Dimer 24. yield: 5.2 mg (41 %); TLC R_f 0.28; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (MALDIFTMS), m/z 889.5571 [M + H^+]

($C_{36}H_{77}N_{10}O_{15}$ requires 889.5564).

Dimer 25. yield: 6.0 mg (51%); TLC R_f 0.26; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (ESI), m/z 843 [M - H^-] ($C_{34}H_{71}N_{10}O_{14}$ requires 843).

Dimer 26. yield: 3.1 mg (28 %); TLC R_f 0.29; (8:2:5:4 ammonium hydroxide-chloroform-ethanol-butanol); (ESI), m/z 859 [M + H^+] ($C_{35}H_{75}N_{10}O_{14}$ requires 859).

Dimer 27. yield: 13.0 mg (90 %); TLC R_f 0.27; (8:2:5:4 ammonium hydroxide-chloroform- ethanol-butanol); (MALDIFTMS), m/z 895.5439 [M + Na^+] ($C_{36}H_{76}N_{10}O_{14}$ requires 895.5435).

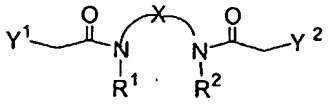
Dimer 28. yield: 8.1mg (64 %); TLC R_f 0.27; (8:2:5:4 ammonium hydroxide-chloroform- ethanol-butanol); (MALDIFTMS), m/z 889.5565 [M + H^+] ($C_{36}H_{77}N_{10}O_{15}$ requires 889.5564).

What is claimed is:

1. A bifunctional antibiotic comprising a first and a second pharmacophore and a linkage for linking said first and said second pharmacophores, each of said first and second pharmacophores having a binding affinity for the A-site of bacterial 16S rRNA sufficient to inhibit translation at clinically effective concentrations, said first and second pharmacophores being identical to one another or different from one another, said linkage having a length and structure for enabling said first and second pharmacophores to bind simultaneously to a single A-site of bacterial 16S rRNA.

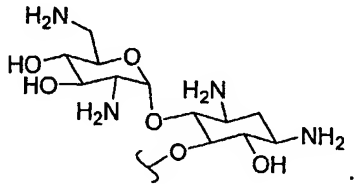
2. A bifunctional antibiotic according to claim 1 wherein at least one of said first and second pharmacophores is inhibitory of APH(2") activity with respect to bifunctional enzyme AAC(6')-APH(2"), the inhibitory activity being sufficient, at clinically effective concentrations, to diminish deactivation of said bifunctional antibiotic by said bifunctional enzyme AAC(6')-APH(2").

3. A bifunctional antibiotic according to claim 1 represented by the following structure:



wherein:

Y^1 and Y^2 are the first and second pharmacophore respectively and are both represented by:

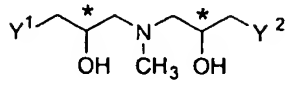


;

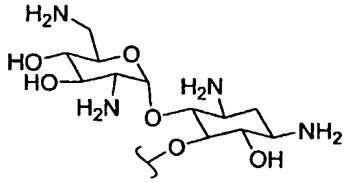
R^1 and R^2 are each independently selected from the group of radicals consisting of -H and $-CH(Ph)CONHCH_2CO_2H$; and

X is the linkage and is selected from the group of diradicals consisting of $-(CH_2)_n-$ and $-[(CH_2)_2O(CH_2)_3]_2O$, where $3 \leq n \leq 12$.

4. A bifunctional antibiotic according to claim 1 represented by the following structure:



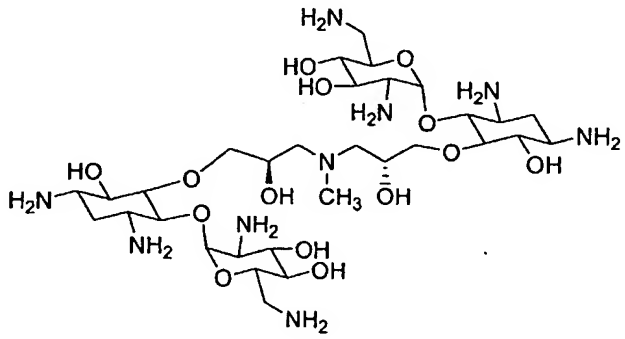
wherein Y^1 and Y^2 are the first and second pharmacophore respectively and are both represented by:



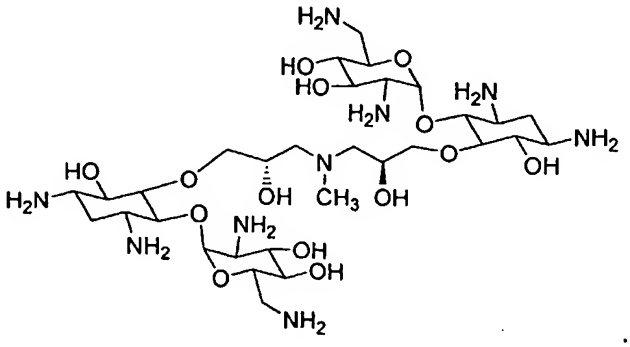
; and

the stereochemistry is either (*S,S*) or (*R,R*).

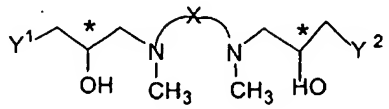
5. A bifunctional antibiotic according to claim 4 represented by the following structure:



6. A bifunctional antibiotic according to claim 4 represented by the following structure:

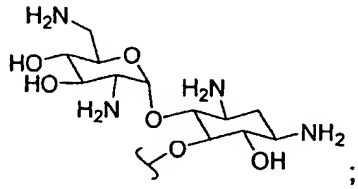


7. A bifunctional antibiotic according to claim 1 represented by the following structure:



wherein:

Y^1 and Y^2 are the first and second pharmacophore respectively and are both represented by:

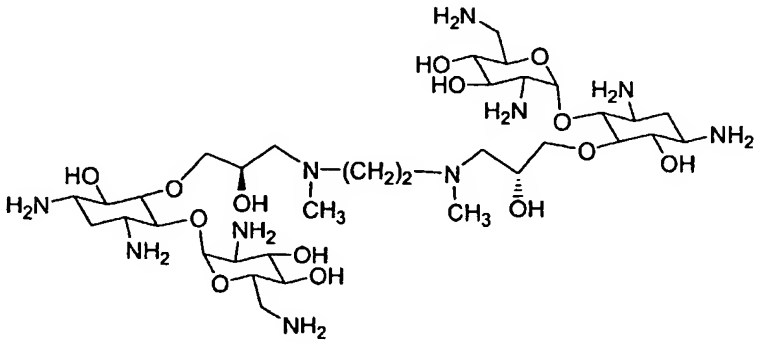


; and

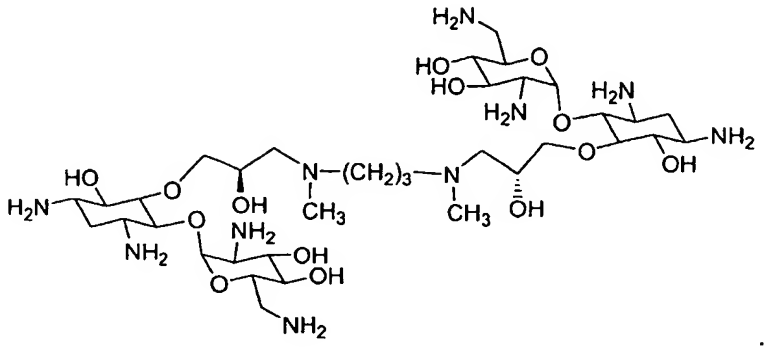
X is the linkage and is selected from the group of diradicals consisting of -$(CH_2)_n$- and -$[(CH_2)_2]_2O$, where $2 \leq n \leq 4$; and

the stereochemistry is either (*S,S*) or (*R,R*).

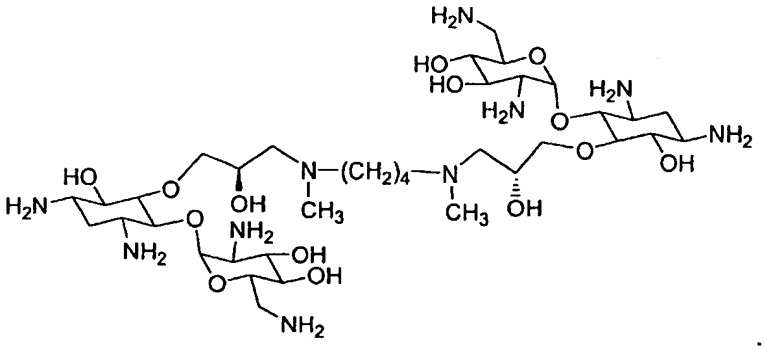
8. A bifunctional antibiotic according to claim 7 represented by the following structure:



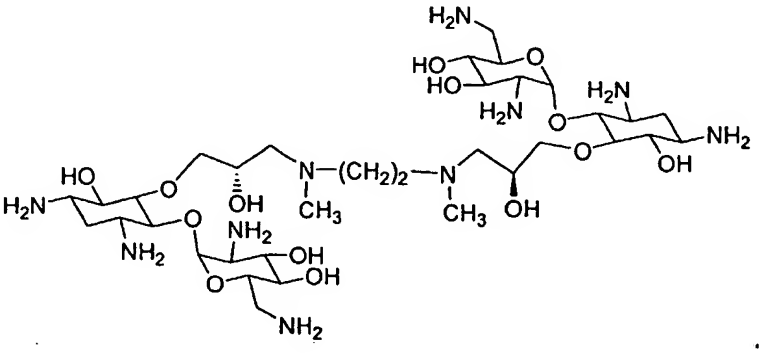
9. A bifunctional antibiotic according to claim 7 represented by the following structure:



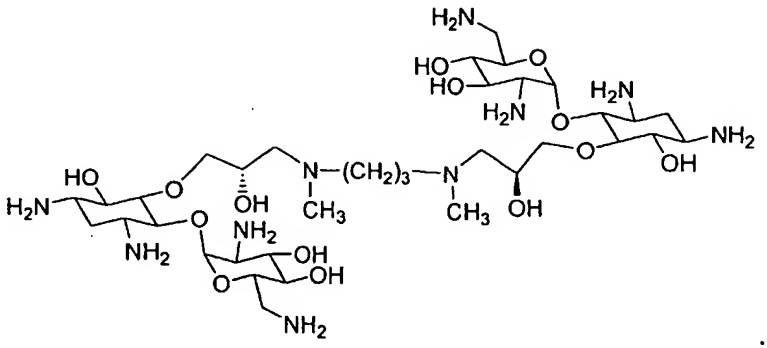
10. A bifunctional antibiotic according to claim 7 represented by the following structure:



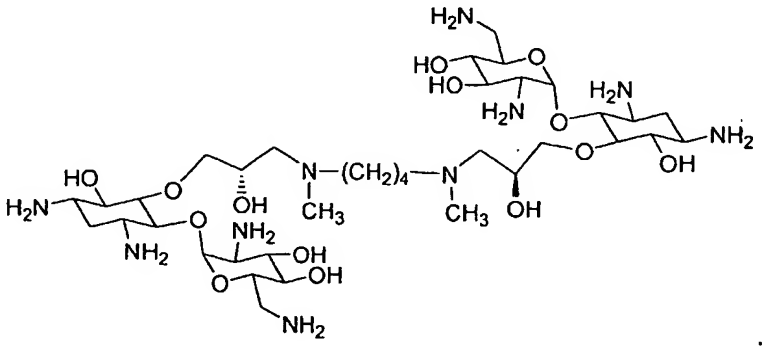
11. A bifunctional antibiotic according to claim 7 represented by the following structure:



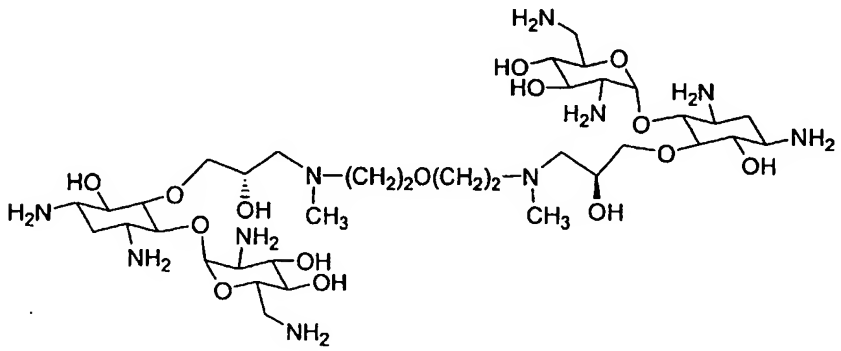
12. A bifunctional antibiotic according to claim 7 represented by the following structure:



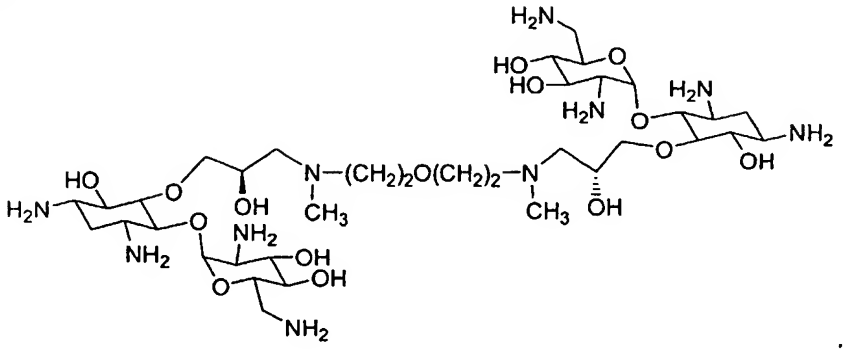
13. A bifunctional antibiotic according to claim 7 represented by the following structure:



14. A bifunctional antibiotic according to claim 7 represented by the following structure:



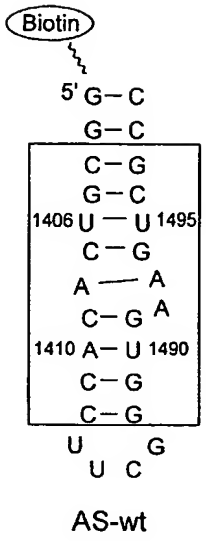
15. A bifunctional antibiotic according to claim 7 represented by the following structure:



16. A bifunctional antibiotic according to claim 1 wherein the first and second pharmacophore are independently selected from the group consisting of neamine, neomycin B, and gentamicin C_1 .

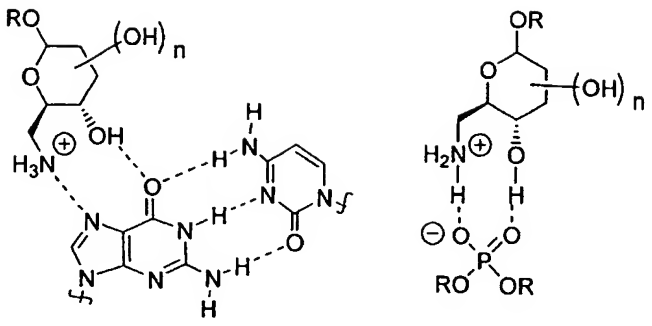
17. A process for inhibiting translation within a bacterium having 16S rRNA with an A-site, said process comprising the step of contacting the bacterium with a concentration of a bifunctional antibiotic selected from claims 1 -16 sufficient to inhibit translation.

18. A process for simultaneously inhibiting translation and APH(2") activity within a bacterium having both 16S rRNA with an A-site and the bifunctional enzyme AAC(6')-APH(2"), said process comprising the step of contacting the bacterium with a concentration of a bifunctional antibiotic selected from claims 1 -16 sufficient to inhibit translation and APH(2") activity.



AS-wt

Figure 1



GC base Pair phosphodiester

Figure 2

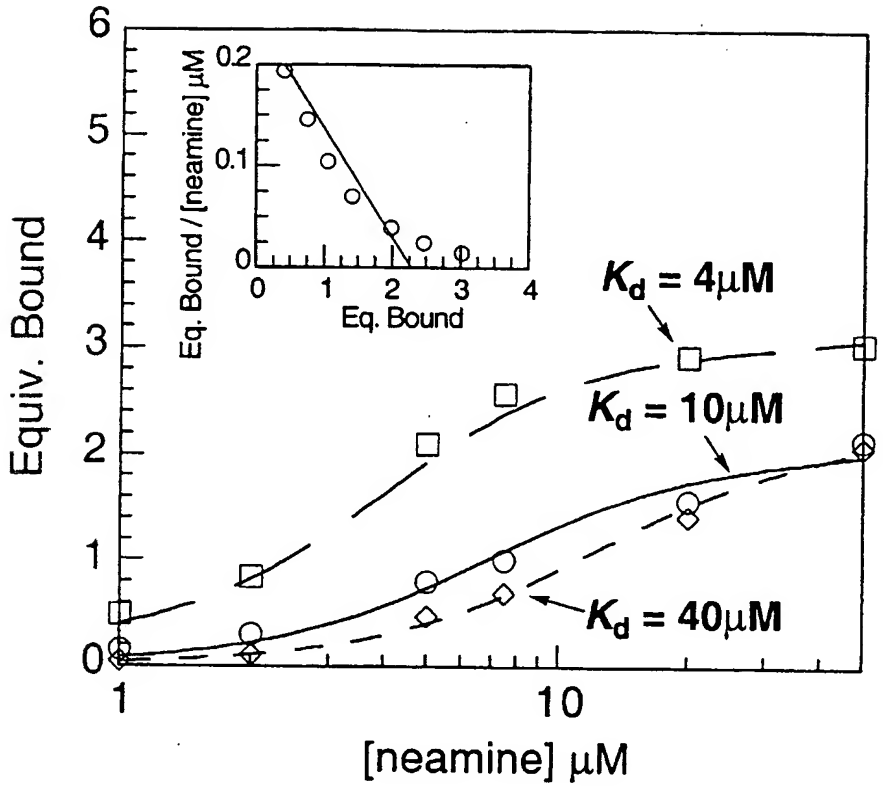
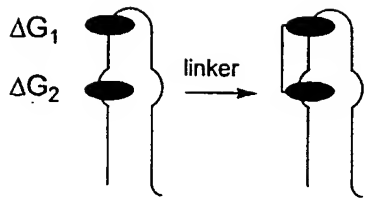

Equiv. Bound
[neamine] μM
Eq. Bound / [neamine] μM
Eq. Bound
K_d = 4μM
K_d = 10μM
K_d = 40μM

Figure 3



$$\Delta G_T = \Delta G_1 + \Delta G_2 + \Delta G_{Linker}$$

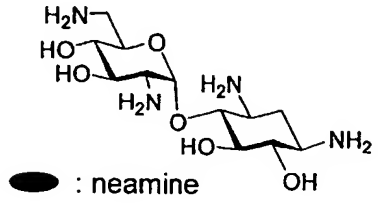
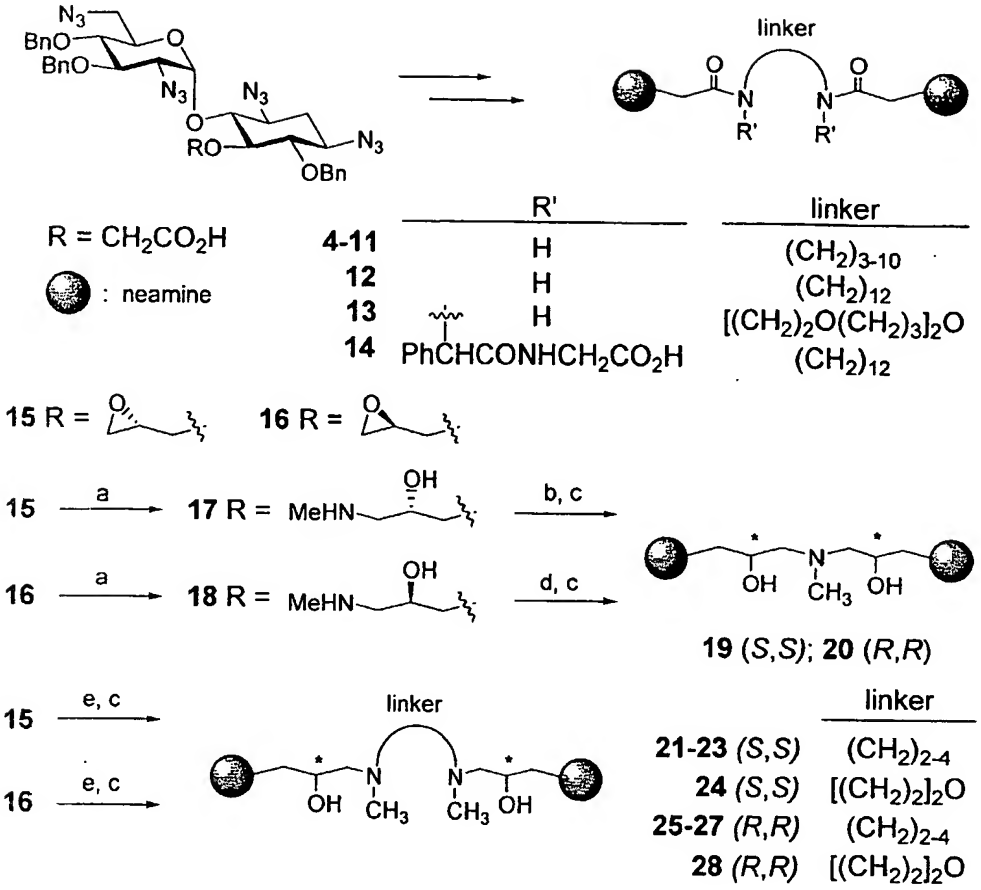


Figure 4



(a) CH_3NH_2; (b) **15**, EtOH, 95 °C, 16 h; (c) (i) $P(CH_3)_3$, THF, H_2O;
(ii) 20 % $Pd(OH)_2/C$ (Degussa), H_2, H_2O, AcOH; (d) **16**, EtOH, 95 °C, 16 h;
(e) diamine, EtOH, 95 °C, 16 h.

Figure 5

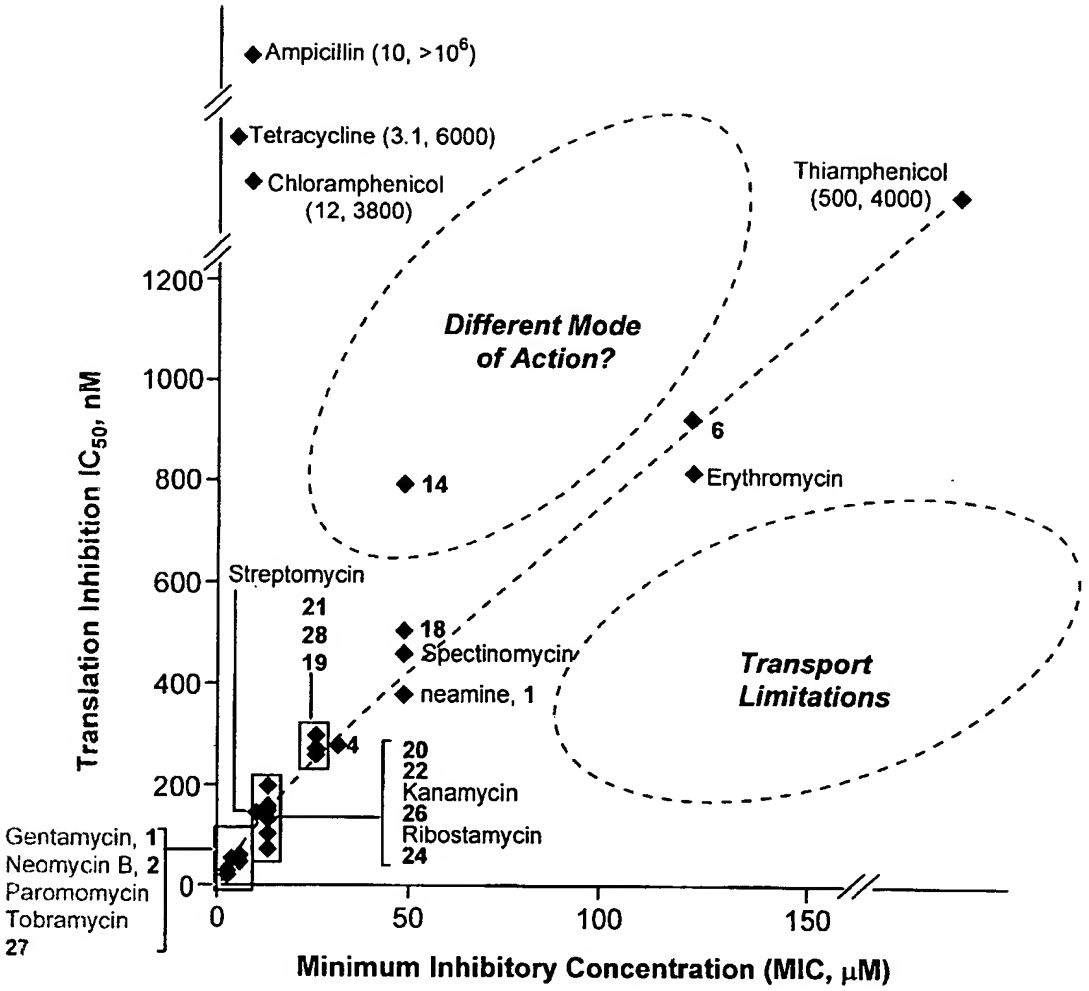
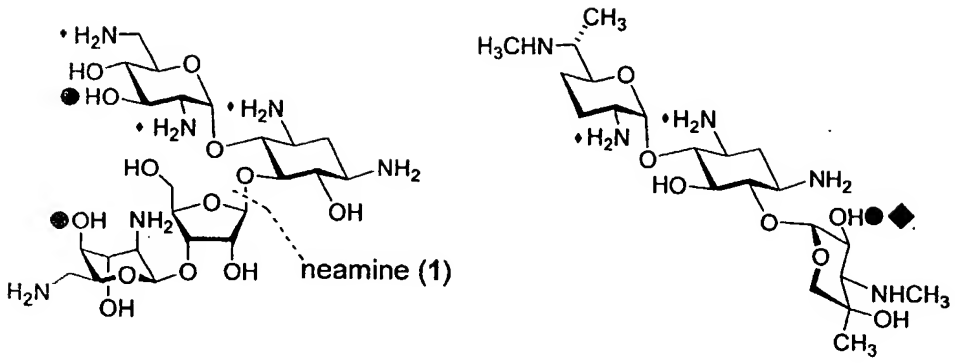

Ampicillin (10, >10^6)
Tetracycline (3.1, 6000)
Chloramphenicol (12, 3800)
Thiamphenicol (500, 4000)
Different Mode of Action?
Transport Limitations
Translation Inhibition IC50, nM
1200
1000
800
600
400
200
0
6
Erythromycin
14
Streptomycin
21
28
19
18
Spectinomycin
neamine, 1
4
20
22
Kanamycin
26
Ribostamycin
24
Gentamycin, 1
Neomycin B, 2
Paromomycin
Tobramycin
27
0
50
100
150
Minimum Inhibitory Concentration (MIC, μM)

Figure 6



neomycin B (2)

gentamicin C_1 (3)

- acetylation
- phosphorylation
- ribosylation

Figure 7

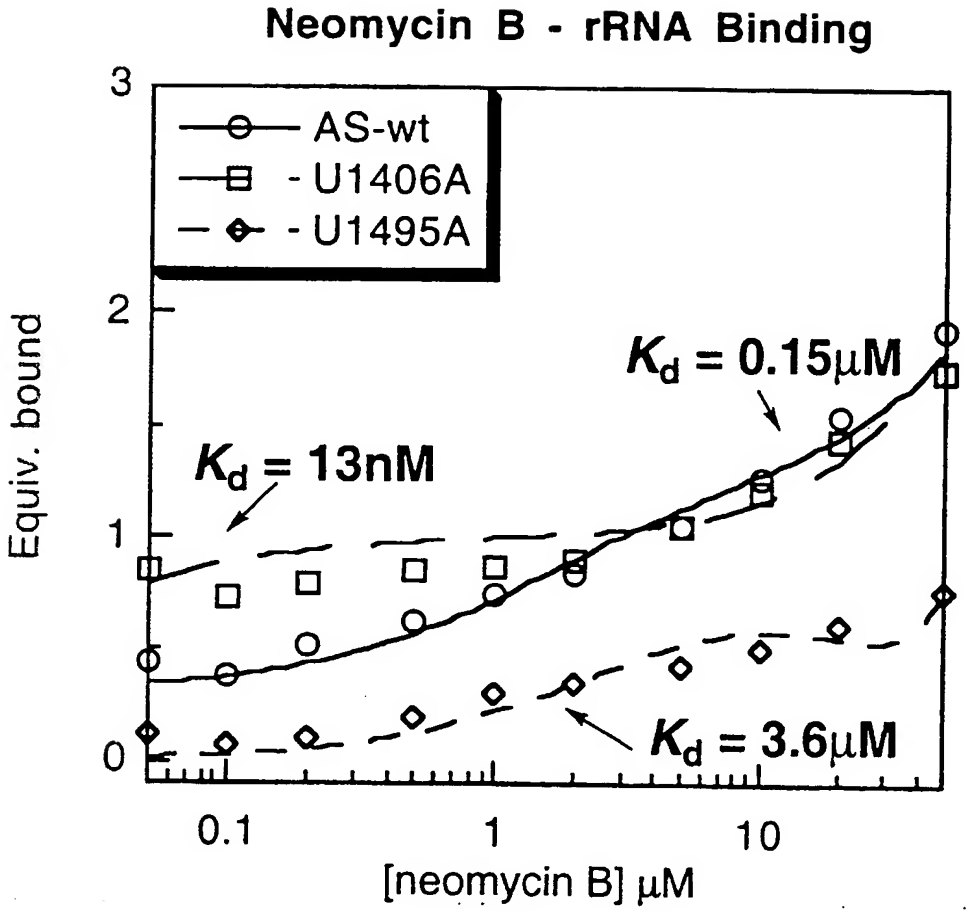

Neomycin B - rRNA Binding
AS-wt
- U1406A
- U1495A
K_d = 0.15μM
K_d = 13nM
K_d = 3.6μM
Equiv. bound
[neomycin B] μM
0
1
2
3
0.1
1
10

Figure 8

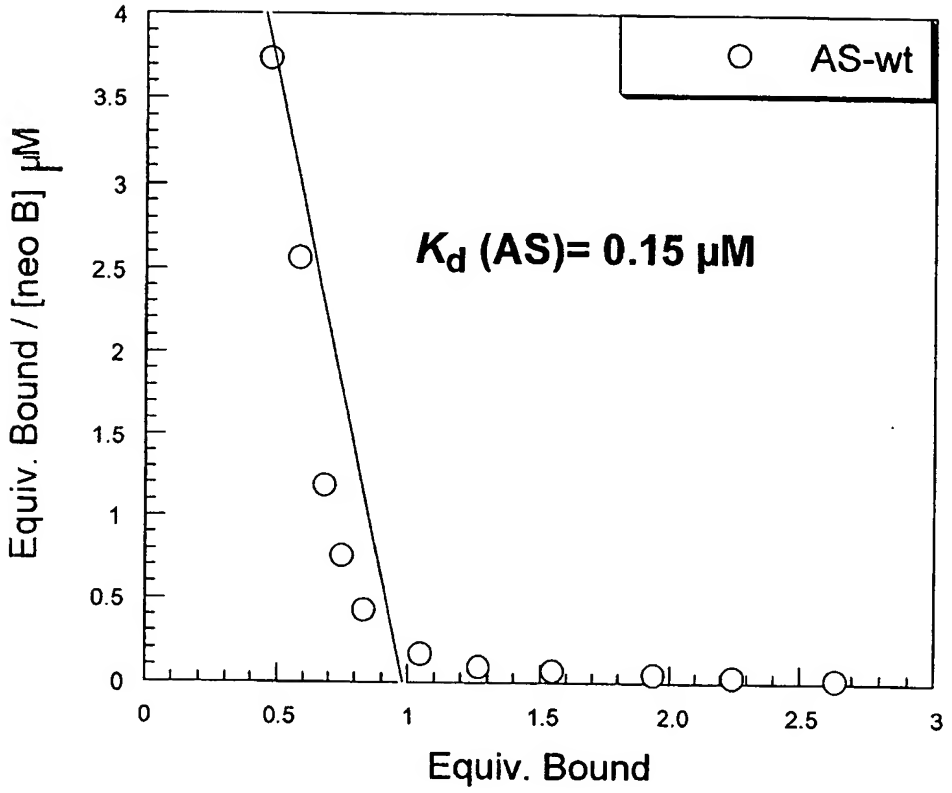

AS-wt
K_d (AS)= 0.15 µM
Equiv. Bound / [neo B] µM
Equiv. Bound
0
0.5
1
1.5
2
2.5
3
3.5
4
0
0.5
1
1.5
2.0
2.5
3

Figure 9

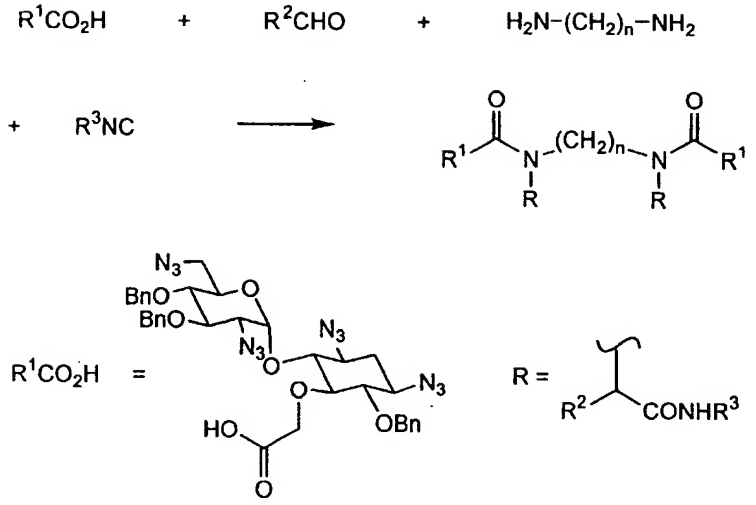


Figure 10

Kirby Bauer Tests: diameters (in mm) of zones of inhibition for test strains. All compounds except neomycin and gentamicin were spotted at 200nmoles/disk; neomycin was spotted at 33nmoles/disk (30μg) while gentamicin was spotted at 10nmol/disk (10μg). SPR K_d values for dimers 4-13 is also provided.

compound	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	K_d (μM)
neamine (1)	17	17	10
neomycin (2)	18.5	21	0.2
gentamicin (3)	19.5	20	1.7
ribostamycin	16.5	14.5	25
paromomycin	18	19.5	0.2
4	14.5	20	1.1
5	14	17.5	4.6
6	12.5	17.5	0.8
7	10.5	14	4.4
8	11	14.5	4.1
9	10.5	14.5	2.3
10	10.5	14.5	2.4
11	13	13.5	2.8
12	10	12.5	1.9
13	11	8.5	21
14	14	14	5.4
17	16	21	12
18	15	20	2.6
19	15	19.5	17
20	18	22.5	1.2
21	15	20.5	0.5
22	16.5	21.5	0.2
23	13	18	5.0
24	18	21.5	1.0
25	11	17.5	0.6
26	17	23	0.8
27	18.5	21.5	0.04
28	16	22	0.8

Figure 11

Minimum Inhibitory Concentration (μM) in *E. Coli* ATCC 25922 and *In Vitro* Translation IC_{50}.

Compound	antibiotic activity (MIC, μM)	translation inhibition (IC_{50}, nM)
neamine (**1**)	50-100	410
neomycin (**2**)	3.1	28
gentamicin (**3**)	1.6	20
ribostamycin	12.5	100
kanamycin	12.5	150
paromomycin	6.25	40
tobramycin	3.1	50
streptomycin	10	150
spectinomycin	50	500
4	31	300
6	125	1000
14	50	860
17	25-50	280
18	50	550
19	25	270
20	12.5	200
21	25-50	310
22	12.5-25	160
23	100	>500
24	12.5	70
25	100	>500
26	12.5	130
27	6.25	55
28	25	280

Figure 12

Kinetic Parameters of Neamine Dimers **6** and Neamine for Various Aminoglycoside Modifying-Enzymes. BF refers to the bifunctional enzyme AAC(6')-APH(2"), where the particular activity tested is indicated.

neamine (1) (Daigle, D. M.; et al. *Chem. Biol.* **1999**, *6*, 99)

Enzyme	K_M	k_{cat} (s^{-1})	K_{is} (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
BF AAC-(6′)	8.40	3.00	15.0	3.6×10^5
BF APH-(2″)	9.6	0.17		1.8×10^4

neamine dimer **4**

Enzyme	K_M (μM)	k_{cat} (s^{-1})	K_{is} (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
BF AAC-(6′)	0.84			
BF APH-(2″)			0.78	
AAC-Ii-(6′)	53.4	0.86		1.6×10^4
APH-(3′)	0.82	0.75		9.14×10^5

neamine dimer **6**

Enzyme	K_M (μM)	k_{cat} (s^{-1})	K_{is} (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
BF AAC-(6′)	4.22	0.967		2.29×10^5
BF APH-(2″)			0.149	
AAC-Ii-(6′)	83.6	1.89		2.26×10^4
APH-(3′)	2.66	0.639		2.49×10^5

neamine dimer **27**

Enzyme	K_M (μM)	k_{cat} (s^{-1})	K_{is} (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
BF AAC-(6′)	0.53			
BF APH-(2″)			0.94	
AAC-Ii-(6′)	28.7	0.26		9.26×10^3
APH-(3′)	1.1	0.42		3.8×10^5

Figure 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/40611

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/70
US CL : 514/25; 536/17.2, 17.9
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/25; 536/17.2, 17.9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUCHECK, S.J. et al. Design of Bifunctional Antibiotics that Target Bacterial rRNA and Inhibit Resistance-Causing Enzymes. Journal of the American Chemical Society. Published on Web May 16, 2000. Vol. 122, pages 5230-5231. See the entire article.	1-18
X	TOK, J.B.-H. et al. Enhanced Binding of Aminoglycoside Dimers to a "Dimerized" A-Site 16S rRNA Construct. Bioorganic & Medicinal Chemistry Letters. July 17, 2000. Vol. 10, pages 1593-1595. See pages 1594-1595.	1, 2, and 16-18
X	WANG, H. et al. Dimeric Aminoglycosides: Design, Synthesis and RNA Binding. Bioorganic & Medicinal Chemistry Letters. 1997. Vol. 7, No. 14, pages 1951-1956. See pages 1951-1952.	1, 2, and 16-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
08 June 2001 (08.06.2001)	13 SEP 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Kathleen Kahler Fonda, Ph.D. Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/40611

Continuation of B. FIELDS SEARCHED Item 3: databases: Registry, HCAPLUS, EAST search terms: claimed structure, antibiotic, amino, glycoside, aminoglycoside, ribosome, ribosomal, rRna

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [63], **Related U.S. Application Data**,

Please replace "Continuation-in-part of application number 08/420,009, filed on April 11, 1995, now abandoned, which is a continuation-in-part of application number 08/110,691, filed on August 23, 1993, now Pat. Num. 5,795,714, which is a continuation-in-part of application number 07/972,012, filed on November 6, 1992; now abandoned and a continuation-in-part of application number 08/419,994, filed on April 11, 1995, now abandoned, which is a continuation-in-part of application number 08/001,323, filed on January 7, 1993, now abandoned." with —Continuation-in-part of application number 08/420,009, filed on April 11, 1995, now abandoned, which is a continuation-in-part of application number 08/110,691, filed on August 23, 1993, now Pat. Num. 5,795,714, which is a continuation-in-part of application number 07/972,012, filed on November 6, 1992; now abandoned.—

Item [57], **ABSTRACT**,
Line 8, please replace "These probe comprise a single-stranded portion" with -- These probes comprise a single-stranded portion --
Item [74], *Attorney, Agent, or Firm*: please replace "Paula Schaeneck" with -- Paula Schoeneck --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Item [56], **References Cited**, U.S. PATENT DOCUMENTS, please add the following references:

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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DATED : August 20, 2002
INVENTOR(S) : Fu et al.

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PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1,
Lines 6-14, replace,

"This application is a continuation-in-part of United States patent application, serial number 08/420,009, filed April 11, 1995, now abandoned which is a continuation-in-part of United States patent application, serial number 08/110,691, filed August 23, 1993, now U.S. patent No. 5,795,714 which is a continuation-in-part of United States patent application serial number 07/972,012, filed November 6, 1992, now abandoned; and United States patent application, serial number 419,994, filed April 11, 1995, now abandoned which is a continuation-in-part of United States patent application, serial number 08/001,323, filed January 7, 1993, now abandoned." with —This application is a continued prosecution application of U.S. Serial No. 08/614,151, filed March 12, 1996, which is a continuation-in-part of U.S. application, Serial No. 08/420,009, filed April 11, 1995, to Cantor, entitled Solid Phase Sequencing of Nucleic Acids, which is a continuation-in-part of U.S. application, Serial No. 08/110,691, filed August 23, 1993, to Cantor et al., entitled Method for Replicating an Array of Nucleic Acid Probes, now U.S. Patent No. 5,795,714, issued August 18, 1998, which is a continuation-in-part of U.S. application Serial No. 07/972,012, filed November 6, 1992, to Cantor, entitled Position Sequencing by Hybridization, now abandoned.—

Lines 16-22, please delete "Rights in the Invention This invention was made with United States Government support under grant number DE-FG-02-93ER61609, awarded by the United States Department of Energy, and the United States Government has certain rights in the invention."

Column 3,
Line 15, please replace "to" with -- too --
Line 31, between "conditions" and "ultimately" please insert -- are --
Line 31, please replace "this s the" with -- this is the --
Line 39, please replace "publication" with -- publications --

Column 6,
Line 33, after "between" please delete "particle the"
Lines 62-63, please remove the paragraph separation between "target constructed." and "Nucleic acids"

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 8,
Line 8, after "fragments" please delete "complexes"

Column 9,
Line 10, please replace "FIG. 13" with -- FIGS. 13A-13J --
Line 20, please replace "FIG. 18" with -- FIGS. 18A-18B --
Line 24, please replace "FIG. 20" with -- FIGS. 20A-20C --
Line 27, please replace "FIG. 21" with -- FIGS. 21A-21D --
Line 29, please replace "FIG. 22" with -- FIGS. 22A-22C --
Line 31, please replace "FIG. 23" with -- FIGS. 23A-23C --
Line 33, please replace "FIG. 24" with -- FIGS. 24A-24C --
Line 52, please replace "be" with -- being --
Line 66, please replace "an" after "be"

Column 10,
Line 13, replace "array" with -- arrays --
Line 45, replace "includes" with -- include --

Column 12,
Line 45, please replace "common one" with -- one common --

Column 15,
Lines 31-34, replace "fluorescein, iododicarbocyanine dye, SiR, $Si(CH_3)_3$, $Si(CH_3)_2(C_2H_5)$, $Si(CH_3)_2(CH_5)_2$, $Si(CH_3)(C_2H_5)_2$, $Si(C_2H_5)_3$, $(CH_2)_nCH_3$, $(CH_2)_nNR$, CH_2CONR, $(CH_2)_nOH$, CH_2F, CHF_2, and CF_3;" with -- SiR_3, $Si(CH_3)_3$, $Si(CH_3)_2(C_2H_5)$,$Si(CH_3)(C_2H_5)_2$, $Si(C_2H_5)_3$, $(CH_2)_nCH_3$, $(CH_2)NR_2$, CH_2CONR_2, $(CH_2)_nOH$, CH_2F, CHF_2, and CF_3; --
Lines 41-45, please replace "the mass-modifying functionality is generated from a precursor functionality which is —N_3 or—XR, wherein X is: —OH, —NH_2, —NHR, —SH, —NCS, —$OCO(CH_2)_nCOOH$, —$NHCO(CH_2)_nCOOH$, —OSO_2OH, —$OCO(CH_2)_nI$, or —OP(O-alkyl)-N-alkyl)$_2$," with -- Mass modifying functionalities, may also be —N_3 or —XR, wherein X is: —O—, —NH—, —NR, —S—, —NHC(S)—, —$OCO(CH_2)_nCOO$—, —$NHCO(CH_2)_nCOO$—, —OSO_2O—, —$OCO(CH_2)_n$—, —OP(O-alkyl)—, —NHC(S)NH—, —$OCO(CH_2)_nS$—, —$OCO(CH_2)S$—, $NC_4O_2H_2S$—, —OPO(O-alkyl)—,— --
Line 55, please replace "acids" with -- acid --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 16,
Line 33, after "nucleic" please insert -- acids --
Line 34, please replace "simply" with -- simple --
Line 40, please delete "contacting by"

Column 18,
Line 21, please replace "5002,868" with -- 5,002,868 --

Column 19,
Line 17, between "have" and successfully" please insert -- been --

Column 22,
Line 21, please replace "well-know" with -- well-known --
Line 43, please replace "5´-temeni" with -- 5´-termini --
Line 43, please replace "3´-temeni" with -- 3´-termini --

Column 23,
Line 47, please replace "small" with -- smaller --
Line 60, please replace "individual" with -- individually --

Column 24,
Line 13, please replace "an array" with -- arrays --

Column 25,
Line 1, please delete "from"
Line 6, please insert -- ordinary -- between "each" and double-stranded"
Line 14, please replace "sequence" with -- sequences --
Line 37, please replace "from" with -- form --
Line 53, after "ladder" please replace "and" with -- which --
Line 54, please replace "sequencing where" with -- sequencing, where --
Line 61, please replace "sequence" with -- sequences --

Column 26,
Lines 6 and 8, please replace " A biotin" with -- Biotin --
Line 14, please replace "reaction This" with -- reaction. This --

Column 27,
Line 35, please replace "used as" with -- used, as --
Line 66, please replace "Any unoccupied" with -- Unoccupied --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 28,
Line 33, please replace "3-aminopropyltrieshoxysilane" with
-- 3-aminopropyltriethoxysilane --

Column 29,
Line 6, please replace "functionality was" with -- functionality, was --

Column 30,
Lines 35-36, please remove the paragraph separation between "double stranded sequencing." and "For single stranded"

Column 31,
Line 19, replace "as then" with -- was then --
Line 51, please replace "discrimination other" with -- discrimination, other --

Column 32,
Line 19, please replace "nucleic also minimize" with -- nucleic acid may also minimize --
Line 24, please replace "method" with -- methods --

Column 34,
Line 31, please replace "**(SEQ ID NO 2)**" with -- **(SEQ ID NO 35)** --

Column 35,
Line 6, please replace "**(SEQ ID NO 2)**" with -- **(SEQ ID NO 35)** --
Line 8, please delete "**(SEQ ID NO 2)**"
Line 51, please replace "FIG. 13" with -- FIG. 13A-13J --

Column 36,
Line 7, please replace "Target TS12" with -- Target TS10 -- in reaction 9
Line 40, please replace "DNA" with -- DNA) --
Line 63, please replace "40 mM $McCl_2$" with -- 40 mM $MgCl_2$ --

Column 37,
Line 23, please replace "(40 mM" with -- 40mM --
Line 32, please replace "5 minutes room temperature" with -- 5 minutes, room temperature --
Line 50, please replace "droplet. the silicon array surfaces is ideal for" with -- droplet. Silicon array surfaces are ideal for --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 38,
Line 41, please replace "Comparison" with -- By comparison --
Line 43, please insert -- , -- between "nucleotide" and "the nucleic acid"

Column 40,
Line 11, please replace "5-(3-aminopropynyl-l)-2´deoxyuridine" with
-- 5-(3-aminopropynyl-1)-2´-deoxyuridine --

Column 43,
Line 14, please replace "bond" with -- bonds --

Column 44,
Line 4, please replace "Aglycyl glycine" with -- A glycyl glycine --

Column 45,
Lines 66-67, please replace "8-glycly– and 8-glycyl-glycyl-2´.3´-dideoxyadenosine-5´ -triphosphates" with -- 8-glycyl– and 8-glycyl-glycyl-2´,3´-dideoxyadenosine-5´ -triphosphates --

Column 46,
Line 9, please replace "15:1685, 1976) and Accounts Chem. Res. 12:204, 1978) and" with -- 15:1685, 1976 and Accounts Chem. Res. 12:204, 1978 and --

Column 47,
Line 18, please replace "d(TAAAACGACGGCCAGUG)" with
-- d(UAAAACGACGGCCAGUG) --
Line 46, please replace "5´-AACAGCTATFACCATG-3" with
-- 5´-AACAGCTATGACCATG-3´ --
Line 65, please replace "incubation" with -- incubated --

Column 48,
Line 15, please replace "paring" with -- pairing --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 49,
Line 6, please replace "(FIG. 18, bottom panel)" with -- (FIG. 18B) --
Line 31, please replace "(FIG. 18, top panel)" with -- (FIG. 18A) --
Line 60, please replace "incubation" with -- incubated --

Column 50,
Line 53, please replace "nucleotides" with -- nucleotide --

Signed and Sealed this

Sixth Day of May, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,436,635 B1
DATED : August 20, 2002
INVENTOR(S) : Fu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,

Item [63], **Related U.S. Application Data**, please replace "Continuation-in-part of application number 08/420,009, filed on April 11, 1995, now abandoned, which is a continuation-in-part of application number 08/110,691, filed on August 23, 1993, now Pat. Num. 5,795,714, which is a continuation-in-part of application number 07/972,012, filed on November 6, 1992; now abandoned." with -- This application is a continuation-in-part of and claims benefit of priority to U.S. Application Serial No. 08/420,009, filed April 11, 1995 (now abandoned). U.S. Application Serial No. 08/420,009 is related to U.S. application Serial No. 08/110,691, filed on August 23, 1993, now U.S. Pat. No. 5,795,714, as a continuation-in-part. U.S. application Serial No. 08/420,009 is a continuation-in-part of U.S. application serial No. 07/972,012, filed on November 6, 1992; now abandoned. --

Column 1,

Lines 6-14, replace "This application is a continued prosecution application of U.S. Serial No. 08/614,151, filed March 12, 1996, which is a continuation-in-part of U.S. application, Serial No. 08/420,009, filed April 11, 1995, to Cantor, entitled Solid Phase Sequencing of Nucleic Acids, which is a continuation-in-part of U.S. application, Serial No. 08/110,691, filed August 23, 1993, to Cantor et al., entitled Method for Replicating an Array of Nucleic Acid Probes, now U.S. Patent No. 5,795,714, issued August 18, 1998, which is a continuation-in-part of U.S. application Serial No. 07/972,012, filed November 6, 1992, to Cantor, entitled Position Sequencing by Hybridization, now abandoned." with -- This application is a continuation-in-part of and claims benefit of priority to U.S. Application Serial No. 08/420,009, filed April 11, 1995 (now abandoned). U.S. Application Serial No. 08/420,009 is related to U.S. application Serial No. 08/110,691, filed on August 23, 1993, now U.S. Pat. No. 5,795,714, as a continuation-in-part. U.S. Application Serial No. 08/420,009 is a continuation-in-part of U.S. application serial No. 07/972,012, filed on November 6, 1992; now abandoned. --

Signed and Sealed this

Ninth Day of September, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office